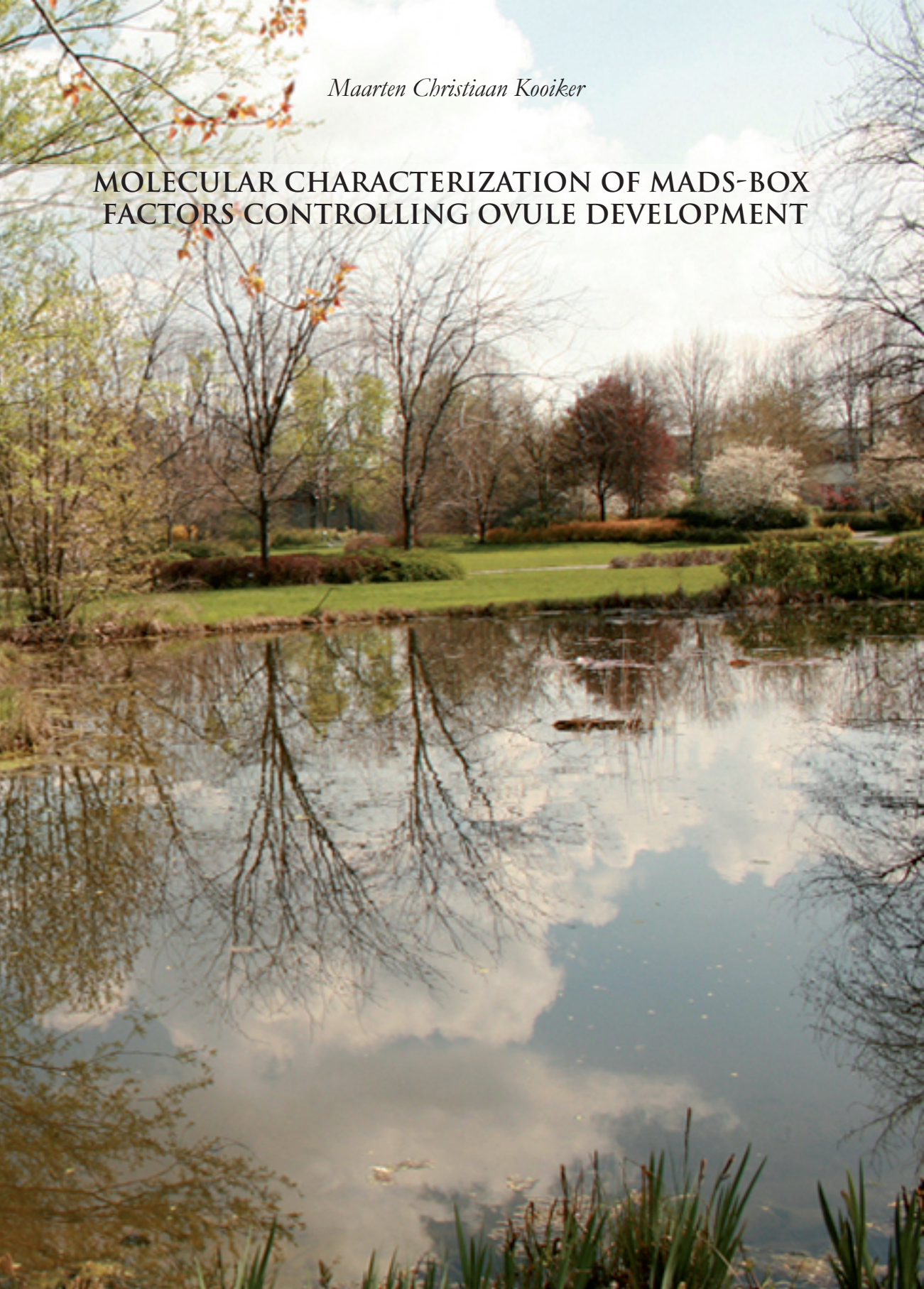
*Maarten Christiaan Kooiker*

# MOLECULAR CHARACTERIZATION OF MADS-BOX FACTORS CONTROLLING OVULE DEVELOPMENT



# Molecular characterization of MADS-box factors controlling ovule development

Maarten Kooiker

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# Molecular characterization of MADS-box factors controlling ovule development

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# Molecular characterization of MADS-box factors controlling ovule development

An academic essay in

Science

Doctoral Thesis

To obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. S.C.J.J Kortmann,
according to the decision of the Council of Deans
to be defended in public on Monday February, 23 2009
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by

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Quant'e bella giovinezza che si fugge tuttavia!
Chi vuol esser lieto, sia: Di doman non c'e certezza.

*Lorenzo de' Medici*

# Contents

# Chapter 1

# Introduction and scope of the thesis

Maarten Kooiker, Raffaella Battaglia, Martin Kater,
and Lucia Colombo

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**ABSTRACT**

Flower development has been intensively studied in Arabidopsis. A considerable number of genes have been identified that control the identity of the floral organs. Almost all the homeotic genes that control floral organ development belong to the same transcription factor family, named the MADS-box gene family. The first Arabidopsis MADS-box gene that was cloned is *AGAMOUS* (*AG*), a gene controlling stamen and carpel development and belonging to a monophyletic clade of MADS-box genes that also include *SHATTERPROOF1* (*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*). These genes together determine carpel and ovule identity as was show in recent studies (Favaro et al. 2003; Pinyopich et al. 2003). Biochemical studies showed that the AG, SHP1, SHP2 and STK proteins can form multimeric complexes, established via the SEPALLATA (SEP) proteins, which have previously shown to act in the formation of MADS-box complexes that control petal and stamen identity. The role of the SEP proteins in ovule development was confirmed by the analysis of *sep* mutants (Pelaz et al. 2000; Ditta et al. 2004). Regulation of the expression of MADS-box genes has been poorly studied, though a few factors that (in-)directly bind the AG regulatory region have been reported (Hong et al. 2003; Bao et al. 2004; Sridhar et al. 2006). I have identified and characterized one putative *STK* regulator named BASIC PENTACYSTEINE1 (BPC1). Previously it has been shown that the BPC proteins regulate the expression of another transcription factor controlling integument development named *INNER NO OUTER* (*INO*) *BPC1* belongs to a small gene family consisting of 7 members that bind to GA-rich sequences, but these genes are not well characterized. In chapter 4 and 5 several experiments are described that will give more insight in the function of these genes.

**INTRODUCTION**

The wild-type Arabidopsis flower consists of four concentric whorls, being from the outside to the inside four sepals, four petals, six stamens and two fused carpels. Flower development has been intensively studied in Arabidopsis. These studies started with the identification of floral homeotic mutants . For example in the mutant *ag* the reproductive organs are affected: the third whorl stamens are replaced by petals and the fourth whorl pistil is replaced by another flower as shown in Figure IB (Bowman et al. 1989; Yanofsky et al. 1990). Mutations in the *APETALA 1* (*AP1*) and *APETALA2* (*AP2*) genes affect the outer two whorls, for example in the *ap2-1* mutant sepals are

converted into carpel-like structures and the petals are converted into stamens (at 25°C) or absent (29°C) (Bowman et al. 1989).

In both *apetala3* (ap3) and *pistillata (pi)* mutants a similar phenotype was observed: the second whorl petals are converted into sepals whereas the third whorl stamens are converted into carpelloid structures (Figure ID) (Bowman et al. 1989).

The observed flower phenotypes in these mutants were used (in combination with similar mutants observed in *Antirrhinum majus*) to formulate a combinatorial model, named the ABC-model, which explains how three classes of genes (class A, B and C) determine the identity of the floral organs (Coen and Meyerowitz 1991). The model proposes that three classes of genes are expressed in adjacent, overlapping whorls such that the A-class is expressed in whorls 1 and 2 and controls sepal and petal identity, the B-class in whorls 2 and 3 controls petal and stamen identity, and the C-class in whorls 3 and 4 determines the identity of stamens and carpels and floral meristem determinacy. Furthermore, the A- and C- classes are mutually antagonistic which means that in the absence of class A expression, class C genes are expressed in the first and second whorl and vice versa.

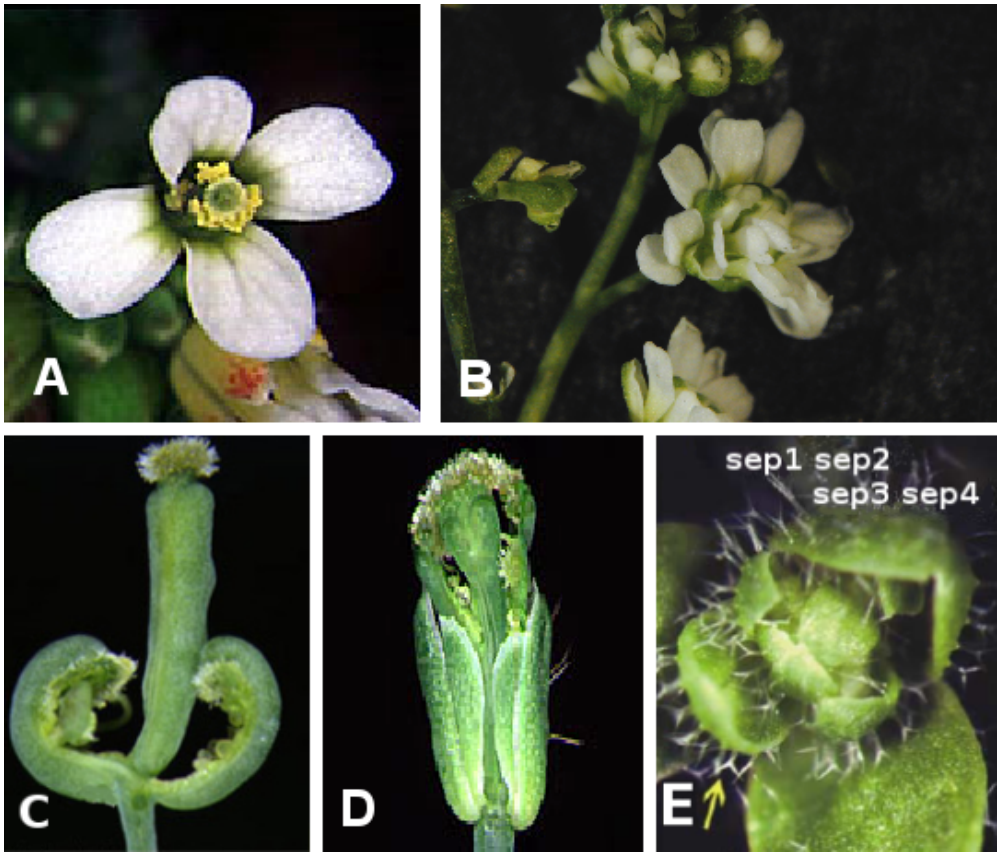


**Figure I:** Phenotype of wild-type and mutant Arabidopsis flowers

**A** wild-type flower

**Figure I**(continued)**:B** *agamous* mutant flower
**C** *apetala2* mutant flower
**D** *pistillata* mutant flower
**E** *sep1 sep2 sep3 sep4* quadruple mutant

The floral homeotic genes responsible for the observed mutant phenotypes have been cloned and belong, except for *AP2*, to the MADS-box gene family (Schwarz-Sommer et al. 1990; Bowman et al. 1991a; Coen and Meyerowitz 1991; Weigel and Meyerowitz 1994).
In 1995 Colombo et al. proposed to extend in Petunia the ABC model with a 4$^{th}$ class, named D genes, where the class D genes (represented by the Petunia *FLORAL BINDING PROTEIN7* (*FBP7*) and *FBP11*) control ovule identity as will be described in more detail later in this chapter. In the year 2000 an extension of this model with class E genes was proposed based on the results obtained in Arabidopsis by Pelaz et al. (2000). They found that when the mutants of the three redundant *SEPALLATA* genes were combined in a triple mutant (*sep1 sep2 sep3*) the inner three whorls loose their identity and are converted into sepals. The flowers of these triple mutants contain only sepals which is identical to the phenotype observed in a class B C double mutant indicating that these *SEP* genes are important for the B- and C-function. Later on a fourth SEP gene (*SEP4*) was added. When the *sep4* mutant allele was combined with the *sep1 sep2 sep3* triple mutant all floral organs were transformed into leaf-like structures (Figure IE; Ditta et al. (2004)). These class E genes complete the current ABCDE model as shown in Figure II.

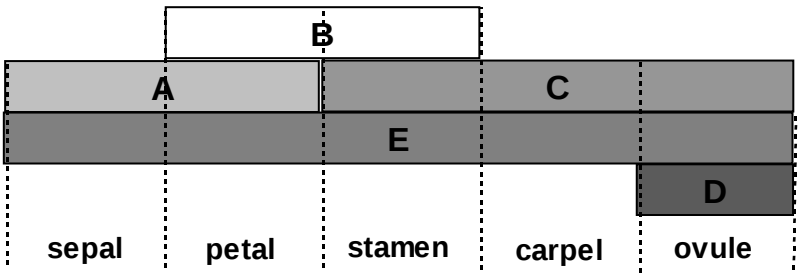


**Figure II:** The ABCDE model in which the activity of different combinations of MADS-box genes determine the identity of the floral organs.

## MADS-BOX GENES CONTROL FLORAL ORGAN DEVELOPMENT

Isolation of the A, B, C, D and E-type genes showed that most of these floral organ identity genes belong to the MADS-box transcription factor family. The acronym MADS is derived from the first

genes that were isolated from this family, namely *MCM1*, *AGAMOUS*, *DEFICIENS* and *SRF*, genes isolated from Yeast, Arabidopsis, Antirrhinum and human, respectively (Schwarz-Sommer et al. 1990). These genes have a highly conserved domain in common, named the MADS-box, which is important for the binding of the protein to the DNA. The best characterized plant MADS-box genes contain besides the MADS-box three other domains namely, the I-region, the K-domain and the C-terminus. The I and K domains are important for MADS-box protein dimerization and the C-terminus, which is the least conserved domain, is important for transcriptional activation and ternary complex formation (Riechmann et al. 1996; Egea-Cortines et al. 1999; Masiero et al. 2002).

The fact that class A, B ,C and D genes as well as the class E genes are essential for the determination of the identity of the floral organs, indicates that there is a genetic interaction between these genes. Further evidence for the genetic interaction between class ABC and E genes came from experiments done by Honma and Goto (2001) and Pelaz et al. (2001a) who ectopically expressed the class A and B genes and the class B and C genes together with class E genes which resulted in the conversion of especially floral organs into petals or stamens, respectively. Rosette and bracts leaves conversion were also observed. Similar experiments done in the past showed that ectopic expression of class A, B or C genes had an effect on the identity of the floral organs in which SEP genes are expressed but not in rosette leaves (Mizukami and Ma 1992; Jack et al. 1994; Riechmann and Meyerowitz 1997). The experiments done by Honma and Goto (2001) showed that the expression of class E genes is needed to be expressed to change the identity of a leaf into a floral organ.

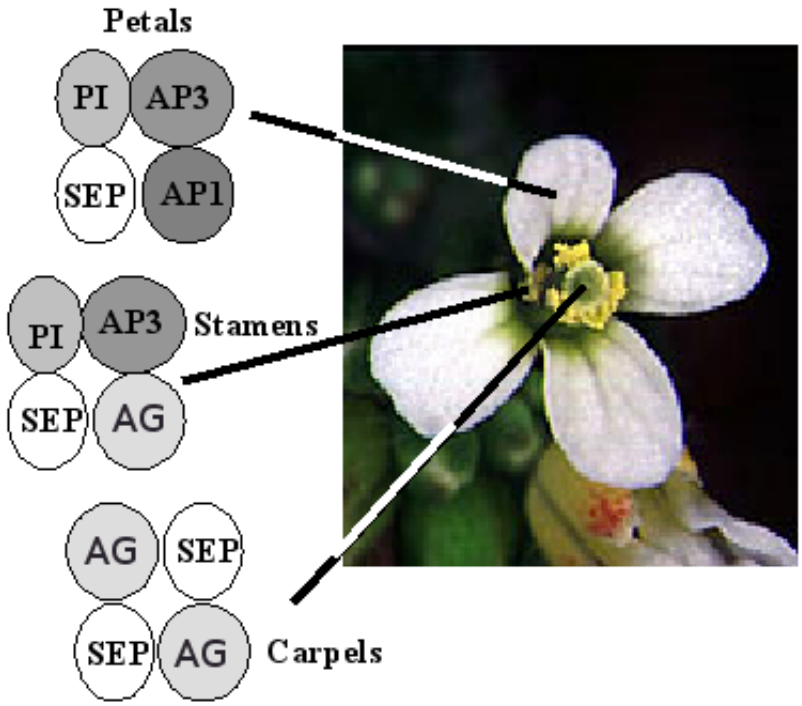


**Figure III: 'Quartet model'.** Model in which protein complexes control the identity of floral organs in Arabidopsis. Petal identity is controlled by the PI, AP3, AP1, SEP protein complex, the identity of stamens is controlled by the PI, AP3, AG, SEP complex and carpel identity is controlled by protein complexes formed by AG and SEP (Theissen and Saedler 2001).

Biochemical studies gave the clue for the observed genetic interactions since they showed that the SEP3 protein (class E) mediates the protein interaction between class A and B MADS-box factors to form petals and between class B and C factors to determine the identity of stamens. As was shown before by Riechmann et al. (1996), the class B MADS-box factors PI and AP3 are able to form dimers, but these proteins by themselves are not able to interact with SEP3, AP1 or AG. However, the PI/AP3 hetero-dimer is able to form ternary complexes with SEP3 and AP1 and is able to form a complex with AG as well in the presence of SEP3 (Honma and Goto (2001); Figure III).

Multimeric MADS-box complexes have first been reported in *Antirrhinum* where the MADS-box proteins SQUAMOSA (SQUA), DEFICIENS (DEF) and GLOBOSA (GLO) form multimeric complex, which is needed for the establishment and maintenance of whorled phyllotaxy (Egea-Cortines et al. 1999).

## THE OVULE IDENTITY GENES

Ovules play an important role in the life cycle of plants, and they have been well studied, both at the morphologic level and at the molecular level. In Arabidopsis ovules are formed inside the carpel, starting as an outgrowth from the placental tissue. In the primordium, three regions can be distinguished, being the funiculus, chalaza and the nucellus.

The funiculus is the connective tissue between the ovule and the mother plant, and is necessary for the transport of nutrients to the ovule. After fertilisation the funiculus cells adjacent to the seed become the abscission zone, which is the point where the seed will be detached from the mother plant. From the chalaza the inner and outer integument will grow asymmetrically to cover the nucellus, leaving a small opening named micropile, where the pollen tube penetrate the embryo sac to release the two sperm cells (Schneitz K. 1995).

In the nucellus the embryo sac develops from a megaspore mother cell, which undergoes meiosis and forms a tetrad. The cell closest to the chalaza is the functional megaspore and will form the embryo sac and the other 3 cells degenerate. This haploid megaspore undergoes a nuclear division to form a two-nuclear embryo sac, which will be separated by a big vacuole. Another nuclear division leads to a cell with four nuclei. The last mitotic division before fertilization, is followed by cellularization, leading to an embryo sac containing three antipodal cells at the chalazal part, two synergid cells next to the egg cell and the central cell, which has a big vacuole and two nuclei, which will fuse to form a diploid nucleus (Figure IV). At the double fertilization both the diploid nucleus and the egg cell fuse with a sperm cell (Schneitz K. 1995).

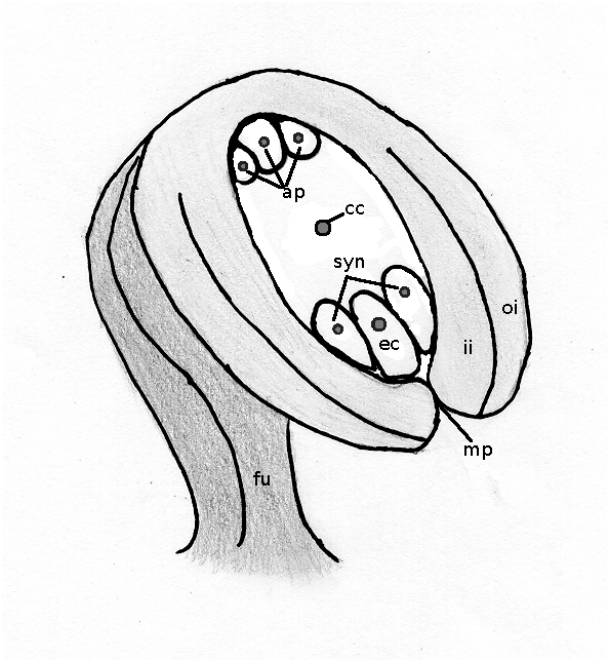


**Figure IV:** Mature Arabidopsis ovule oi, outer integument; ii, inner integument; mp, micropyle; fu, funiculus; syn, synergids; ec, egg cell; cc, central cell (dihaploid); ap, antipodal cells.

At the molecular lever, many genes have been identified that play an important role in the development of ovules. In Petunia for example the co-suppression of the MADS-box genes *FLORAL BINDING PROTEIN7 (FBP7)* and *FBP11* resulted in the formation of carpel-like structures directly on the placenta, replacing the ovules (Angenent et al. 1995). Ectopic expression of *FBP11* resulted in the formation of ovules on sepals and petals. As mentioned before these results lead to the extension of the ABC model to the ABCD model, where the D genes (*FBP7/11*) are responsible for the ovule identity. Similar results were obtained when *FBP2* and *FBP5* (SEP-like genes) were knocked out, resulting in the conversion of ovules into leaf-like structures (Ferrario et al. 2003; Immink et al. 2003).

In Arabidopsis the knock-out of the *SEP*-genes results, as described before, in the conversion of floral organs into leafs. However titration experiments using *SEP1/sep1 sep2 sep3* mutants resulted in the conversion of ovules into carpel-like structures, showing the importance of the *SEP* genes in the ovule formation, as described in more detail in chapter 3. Similar results were obtained knocking out the ovule identity genes *STK*, *SHP1* and *SHP2*, resulting in the conversion of ovule integuments into carpeloid structures (Pinyopich et al. 2003; Brambilla et al. 2007). In chapter 3 it is shown that there is both a physical interaction and genetic interaction between the SEP proteins and the ovule identity proteins, where *SEP1/sep1 sep2 sep3* mutants show a similar phenotype as

the *shp1/2 stk* triple mutant.

Recently, Brambilla et al. (2007) showed that the ovule identity complex (STK, SHP1/2 and SEP proteins) plays an important role in the determination of integument identity and in the stabilisation of the complex formed between AG, BEL1 and SEP proteins (for a review see (Colombo et al. 2008a).

This latter complex seems to have at least two fuctions: the repression of *WUS*, which is normally expressed in the nucellus (Gross-Hardt et al. 2002). In fact in the *bel1* mutant and *bel1 stk shp1 shp2* quadruple mutant the expression of *WUS* is extended to the chalazal and funiculus regions of the ovule (Brambilla et al. 2007). However, whereas ectopic expression of *WUS* normally leads to the formation of several outer integument primordia, the mutants described above show only one enlarged integument-like structure. *STK* is expressed in this structure suggesting that it has integument identity, but the outer integument marker *INNER NO OUTER* (*INO*) is not expressed. The expression of these two genes and the lack of multiple integument primordia, suggest that the enlarged structure that develop from the chalaza in the *bel1* mutant has an inner integument identity at least at early stages of development (Brambilla et al. 2007).

Though the ovule identity complex plays a central role in the development of the ovule, several other genes have been identified that are involved in it's development. One of the first events in ovule formation is the proximal-distal patterning, which is affected in the *sporocyteless (spl)* or *nozzle* (*nzz*) mutants, resulting in multiple defects in ovule development (Yang et al. 1999; Balasubramanian and Schneitz 2000). Besides that, the *spl* mutant also affects the normal development of the nucellus (Yang et al. 1999). Other genes important for the development of integuments are *WUS* (as described above; Gross-Hardt et al. (2002), *AINTEGUMENTA* (*ANT*) (Elliott et al. 1996; Klucher et al. 1996) and *INO* (Villanueva et al. 1999). Whereas *WUS* and *ANT* play an important role in the initiation of both integuments, *INO* plays an important role in the growth of the outer integument and in fact, severe *ino* mutants lack outer integuments. This gene is not only regulated by *ANT* and *BEL1* (Balasubramanian and Schneitz 2000), but also by an other transcription factor, namely BASIC PENTACYSTEINE1 (BPC1) (Meister et al. 2004). BPC1 was identified as a protein that binds to GA repeats and is a member of a small gene family of 7 members in Arabidopsis. The protein has a C-terminal DNA-binding domain that contains several basic amino acid residues and 5 cystein residues, that are highly conserved, even between different plant species (hence the name BASIC PENTACYSTEINE)(Meister et al. 2004). The *BPC* genes are expressed throughout the entire plant and are located in the nucleus, as was shown by Meister et al.

with transient expression experiments in onion epidermal cells. In chapter 4 it is shown that *BPC1* does not only regulate *INO*, but also the ovule identity gene *STK*.

## Regulation of MADS-box genes

After sequencing of the entire Arabidopsis genome in 2000 (Arabidopsis_Genome_Initiative 2000), 107 members of the MADS-box gene family were identified and cloned (Parenicová et al. 2003). Most of the MADS-box genes that have been studied until now, belong to the MIKC sub family, however those with a known function account for less than 20% of all the isolated MADS-box genes. Loss of function mutants have been described for several genes and it is clear that MADS-box genes are involved in various developmental pathways. As previously mentioned, the identity of the floral organs are controlled by MADS-box genes, but also other developmental processes are controlled by MADS-box genes, like the development of the female gametophyte and biogenisis of organelles during embryo development is controlled by *AGL23* (Colombo et al. 2008b) and seed pigmentation is known to be controlled by *TRANSPARENT TESTA16* (Nesi et al. 2002). *AGL62* plays an important role in the cellularization of the endosperm after fertilisation (Kang et al. 2008) and AGL61 and AGL80 are required for the central cell formation (Bemer et al. 2008; Steffen et al. 2008).

Besides the control of sepal and petal identity, *AP1* also controls the determination of floral meristem identity (Mandel et al. 1992), together with *CAULIFLOWER* AGL24 and SVP (Bowman 1993; Gregis et al. 2008) Another intensively studied developmental pathway in Arabidopsis is the floral transition, which is controlled by many MADS-box genes, and in particular *FLOWERING LOCUS C (FLC), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), SVP* and *AGL24* play a central role in the flowering time (for a review see: Henderson and Dean (2004)). Strikingly, several genes are involved in different pathways like, as described above, *AP1* which is involved in the determination of floral meristem identity and the determination of the identity of the first two floral organs, sepals and petals.

Also *AGL24* and *SVP* are two genes that are involved in different pathways, being the regulation of flowering time and they regulate AG during early stages of flower development. Whereas *AGL24* is a promoter of flowering time (Yu et al. 2002; Michaels et al. 2003), *SVP* on the contrary is a repressor of flowering time (Hartmann et al. 2000). Though the two genes show an elevated homology (Parenicová et al. 2003), they have opposite functions with respect to flowering time.

However at later stages they cooperate in the repression of the floral homeotic gene *AG* by forming multimeric complexes with AP1, LEUNIG (LEU) and SEUSS (SEU) (Gregis et al. 2006). LEU and SEU have been shown to form a repressive complex and interact in vitro and in vivo (Sridhar et al. 2004) and together they repress *AG* (Franks et al. 2002). The interaction of these proteins is evidenced more in vivo by Gregis et al. (2006), by the triple mutant *agl24 svp ap1*, which have a severely enhancement with respect to the single and double mutants. The repressive dimer of SEU and LEU has been shown to interact with the AP1/SEP3 complex by (Sridhar et al. 2006), who showed with co-immuno precipitations that LEU/SEU binds to the *AG* regulatory region.
This shows that MADS-box proteins can fulfil multiple functions by forming complexes with different partners, and therefore an interaction map of all MADS-box proteins can give important clues about the functions of the many MADS-box genes with an unknown function as well as new functions for already characterized MADS-box genes. To obtain these results a large scale two-hybrid analysis was performed by de Folter et al. (2005), as described in chapter 2.
As mentioned above, several genes have been shown to bind the *AG* regulatory region, most of which can be found in the second intron. Besides the LEU/SEU complex which binds the *AG*-promoter indirectly, also BELLRINGER (BLR) has been shown to bind directly to the *AG* regulatory region by electro mobility shift assays (EMSA). *BLR* is necessary to prevent *AG* expression in the first two whorls of the flower (Bao et al. 2004). Furthermore binding sites for WUS and LFY can be found in the 3kb second intron of *AG* (Hong et al. 2003). Other genes that are known to regulate *AG* are *CLF* and *ATX1*, Arabidopsis homologs of *Enhancer of Zeste (E(z))* and *Trithorax (TRX)* respectively. Mutants for these two genes affect the chromatin state of *AG* (Saleh et al. 2007). TRX and E(z) belong to the TRITHORAX Group (TRG) and POLYCOMB GROUP (PCG) of proteins that are known to activate and repress homeotic genes in Drosophila and mammalians (Lehmann 2004). This regulation is achieved by the modulation of the histon code on the homeotic genes. It is very interesting to see that in Arabidopsis, the ovule identity gene *STK* is regulated by BPC1 (see chapter 4 and 5), a protein that has several characteristics in common with GAGA FACTOR (GAF) (encoded by the gene *TRITHORAX LIKE* (*TRL*)(Benyajati et al. 1997)), like binding to GA-rich sequences and regulation of homeotic genes.

**Scope of the thesis**

The aim of the research described in this thesis is the molecular understanding of the function of the ovule identity MADS-box gene *SEEDSTICK* (*STK*) and it's regulation.
In several organisms it has been shown that MADS-box proteins form dimers and multimeric complexes. In Arabidopsis these complexes have shown to be sufficient to change organ identity. The complex formed between B- C- and E-type proteins for example have shown to be sufficient to transform cauline leafs into staminoid organs and all floral organs into stamens or staminoid organs (Honma and Goto 2001). In **chapter 2** a matrix based yeast 2-hybrid screening between most of the 107 MADS-box proteins is presented. The results indicate in which developmental pathway some of the uncharacterized MADS-box proteins might act. Furthermore does the dimerization of proteins involved in flower induction and floral organ identity indicate cross-talk between these developmental programs mediated by the dimerization of these MADS-box proteins.
Other interesting interactions that were found are the interactions between the SEP proteins and other proteins of the ABCD model described in the introducion. In **chapter 3** yeast two-and three hybrid experiments show the formation of higher order complexes between the ovule identity proteins STK, SHP1, SHP2, AG and the SEP proteins. These results indicate that the ovule identity is determined by the formation of higher order complexes, as was already shown to be the case for the floral organs. Genetic data supported this hypothesis, since *stk shp1 shp2* triple mutants show a similar phenotype as the *SEP1/sep1 sep2 sep3* mutant plants.
Furthermore it was shown that ectopic expression of *STK* and *SHP* leads to the conversion of sepals (and in some cases bracts) into carpel-like structures, showing the ability of these genes to induce the carpel pathway, even in the absence of *AG*.
The experiments described above show the importance of a tight regulation of these transcription factors, since ectopic expression and knockout mutants of these ovule identity genes lead to the homeotic conversion of floral organs. However, in Arabidopsis little is known about the regulation of homeotic genes. Some genes are known to be regulated by binding of transcription factors to intragenic regions, like for example *AG* that is regulated by BELLRINGER (BLR), LEAFY (LFY), SVP and AGL24 that are able to bind the second intron of *AG*.
Also in the case of the ovule-specific MADS-box gene *STK*, the first intron showed to be of great importance to maintain it's ovule specific expression. In order to get more insight in the regulation of *STK*, in **chapter 4** a one-hybrid screening using the regulatory region of *STK* is described. One of

the proteins that was able to bind to several fragments of the regulatory region of *STK* was BPC1, a factor that has been shown to bind to purine rich sequences. BPC1 is a member of a small protein family consisting of 7 members, which have a highly conserved C-terminus, which is basic and contains 5 highly conserved cysteine residues, hence the name Basic PentaCysteine (BPC). These proteins have all been shown to bind purine rich sequences and on basis of sequence homology can be divided in three classes, class 1 containing BPC1-3, class 2 containing BPC4-6 and BPC7 being the only member of class 3 (Meister et al. 2004).

Analysis of *bpc1* mutants did not reveal striking phenotypes, though the expression of *STK* was slightly altered in these mutant plants. The lack of phenotypes might be caused by redundancy between the members of class 1 genes *BPC1-3*.

In **chapter 5** a functional analysis of the type I *BPC* sub-family is described. A part of the ovules formed in *bpc1 bpc2 bpc3* triple mutants show severe defects in ovule development. Investigation in the cause of these defects is at this moment still in progress.

Another strategy to determine the function of the *BPC* genes that is described in **chapter 5** is the expression of these genes under the 35S constitutive promoter. In these plants the late arising flowers show an ap2-like phenotype. real-time RT experiments showed an increase in *AG* expression and a decrease in *AP2* expression in these plants. Crossings these plants with *ag3* heterozygous plants will show the involvement of *AG* in these mutants.

# Chapter 2

# Comprehensive Interaction Map of the Arabidopsis MADS Box Transcription Factors

Stefan de Folter, Richard G.H. Immink, Martin Kieffer, Lucie Pařenicová, Stefan R. Henz, Detlef Weigel, Marco Busscher, Maarten Kooiker, Lucia Colombo, Martin M. Kater, Brendan Davies, and Gerco C. Angenent

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**ABSTRACT**

Interactions between proteins are essential for their functioning and the biological processes they control. The elucidation of interaction maps based on yeast studies is a first step toward the understanding of molecular networks and provides a framework of proteins that possess the capacity and specificity to interact. Here, we present a comprehensive plant protein–protein interactome map of nearly all members of the Arabidopsis thaliana MADS box transcription factor family. A matrix-based yeast two-hybrid screen of >100 members of this family revealed a collection of specific heterodimers and a few homodimers. Clustering of proteins with similar interaction patterns pinpoints proteins involved in the same developmental program and provides valuable information about the participation of uncharacterized proteins in these programs. Furthermore, a model is proposed that integrates the floral induction and floral organ formation networks based on the interactions between the proteins involved. Heterodimers between flower induction and floral organ identity proteins were observed, which point to (auto)regulatory mechanisms that prevent the activity of flower induction proteins in the flower.

**INTRODUCTION**

Biological processes are executed by proteins that, to a large extent, depend on interactions with other proteins for their activity. These interactions are specific, even among members of a particular protein family that contain similar interaction domains, and are often maintained during evolution. Studying these specific interactions reveals networks of molecules that may lead to potential functional linkages and molecular explanations of biological processes in an organism. These networks are complex, highly dynamic in place and time, and far from understood. The elucidation of interaction maps based on in vitro or yeast studies is a first step toward the understanding of molecular networks and provides a framework of proteins that possess the capacity and specificity to interact. Many recent reports have presented large-scale interaction network maps from Saccharomyces cerevisiae (Uetz et al. 2000; Ito et al. 2001), Caenorhabditis elegans (Walhout et al. 2000), Drosophila melanogaster (Giot et al. 2003), Mus musculus (Suzuki et al. 2001), and humans (Lehner and Fraser 2004) using yeast two-hybrid assays or affinity purification followed by mass spectrometry (Link et al. 1999; Gavin et al. 2002; Ho et al. 2002). Surprisingly, comparable data sets from yeast, for example, revealed hardly any overlap in interactions, suggesting that each

approach provides a subset of the interactome (Von Mering et al. 2002; Bader et al. 2004). Furthermore, these reports demonstrated that two-hybrid data are reliable when several validation criteria are used. Information about interactions of orthologous proteins in other species is informative and may help in validating the interaction data. The conservation of these so-called interologs has been revealed between yeast and bacteria (Kelley et al. 2003) but also between different plant species (Favaro et al. 2002). Previously, we have demonstrated that many interactions between MADS domain proteins are conserved among Arabidopsis thaliana, rice (Oryza sativa), petunia (Petunia hybrida), and Antirrhinum majus (Immink and Angenent 2002). Another criterion for the validation of the interaction data is the colocalization of the interacting proteins in a particular cell. Several studies reported the coevolution of expression of interacting proteins and their ability to physically interact (Ge et al. 2001; Immink et al. 2002; Fraser et al. 2004). This provides a tool to validate interaction data but can also be useful to predict novel protein–protein interactions. Furthermore, other functional genomic or genetic data, such as mutants, may provide additional evidence for the in vivo existence of a particular interaction. By zooming in on a particular group of proteins that is known from previous studies to be enriched for interactions, insight into individual pathways can be obtained. Transcription factors are an interesting class of proteins in this respect. Dimerization of transcription factor proteins increases the selectivity of protein– DNA interactions and creates a large number of diverse DNA binding complexes from a relatively small number of proteins. The gene family encoding MADS domain transcription factors in plants encompasses a relatively large family with 107 members in the Arabidopsis genome (Parenicová et al. 2003). They are further subdivided into two groups: the class II MADS box proteins, comprising the MIKC and Mδ types, and the class I proteins that are further subdivided into the Mα, Mβ, and Mγ types (Alvarez-Buylla et al. 2000a; Parenicová et al. 2003). A wealth of genetic and functional information is available from the MIKC group, whereas the type I subfamily with ; 60 members represents a virtually unknown group of transcription factors. Many MIKC proteins are active in a combinatorial manner to specify the identity of organs (Coen and Meyerowitz 1991). Recent genetic and yeast two- and three-hybrid studies revealed that these MADS box proteins are able to form multimeric complexes (Honma and Goto 2001) and as hypothesized in the quartet model as tetrameric complexes (Theissen and Saedler 2001). These higher-order complexes are supposed to be composed of two dimers that interact at the C termini (Egea-Cortines et al. 1999). Nevertheless, information about MADS protein interactions is limited for Arabidopsis and lacks any data on the type I proteins. Besides Arabidopsis, MADS-dimerization

patterns have been reported for several species, including petunia, rice, Chrysanthemum Dendrathema grandiflorum, and Antirrhinum (Davies et al. 1996; Egea-Cortines et al. 1999; Favaro et al. 2002; Immink et al. 2003; Shchennikova et al. 2004), which provided data for comparative studies and revealed interactions between orthologous proteins. Here, we report a comprehensive plant interactome map of nearly all members of the Arabidopsis MADS box family. It reveals interactions between type I, type II, and between the two types of proteins. Combined with phylogenetic analysis, it sheds light on evolutionary aspects of this protein family. Clustering of proteins based on their interaction pattern pinpoints proteins involved in the same developmental program and provides evidence for the participation of uncharacterized proteins in these programs. Finally, we propose a model that integrates the network of floral induction proteins with the network of floral organ identity proteins, and we predict feedback loops between the two sub-networks.

## RESULTS

### Comprehensive Analysis of MADS Box Transcription Factor Dimerization

Several studies with various plant species have revealed that MADS domain transcription factors form specific homodimers and heterodimers. In general, individual screens of cDNA expression libraries with the yeast two-hybrid GAL4 system have been used for this purpose. These assays are laborious, they result in the identification of a relatively high number of false positives, and they are often limited because of autoactivation of yeast reporters by the presence of an intrinsic activation domain in the bait protein. Therefore, in this study, a matrix-based yeast two-hybrid approach has been followed to identify specific dimerization among the members of the Arabidopsis MADS domain transcription factor family. The complete data set with all the scores is presented in Supplemental Table 1 online, and the interactions are summarized in a matrix in Figure 1 and in Supplemental Table 2 online. Remarkably, the MIKC proteins that contain the K-box, a domain specific for type II plant MADS box proteins that is presumed to fold into an amphipathic a-helical structure (Riechmann and Meyerowitz 1997; Alvarez-Buylla et al. 2000a), interact preferably with other type II proteins and hardly form dimers with the type I MADS box proteins. However, there are some exceptions. In particular, there is a preference for interactions with type I proteins from the Ma subclade. Among the type I proteins, most heterodimers are found between members of different subclades. Interactions among Ma proteins are rare, but they dimerize preferentially with

many proteins of the Mb and Mg clades. Similarly, only a few interactions among members of the Mb and Mg clades were observed, and Mb-Mg heterodimers are rare. This suggests that the participation of a Ma protein is a prerequisite for a stable dimer consisting of only type I proteins. Although many interactions were observed, a relatively large number of MADS domain proteins appeared to have no interactions at all. Possibly these proteins interact only with non-MADS box proteins, or alternatively, particular interactions are not formed in a yeast two-hybrid assay. For example, the interaction between the B-type proteins APETALA3 (AP3) and PISTILLATA (PI) was not found in this screen. Previously, these proteins appeared to interact exclusively in a higher-order complex, with either SEPALLATA3 (SEP3) or AP1 (Honma and Goto 2001), suggesting that the additional factors stabilize the AP3-PI dimer. This requirement for stabilizing factors to maintain specific dimers could be more general. Homodimerization is another form of MADS domain transcription factor interaction that is difficult to detect by yeast two-hybrid analysis (Immink and Angenent 2002), and hence, many homodimers have probably been missed in this screening. Subsequently, the proteins were clustered based on the obtained interaction patterns, which allows the identification of proteins with similar interactions and groups of proteins that are highly connected (Figure 2). This analysis gives clues about the involvement of proteins in certain developmental programs. It reveals groups of proteins with common known functions, but more informatively, also shows clusters containing uncharacterized proteins, for which a function can now be predicted, based on their presence in a particular interaction cluster.

**Data Validation of Yeast Two-Hybrid Experiments**

To obtain more insight into the reliability of the data, a comparison was made between our interaction data and Arabidopsis MADS domain protein interactions described in the literature. In contrast with the wealth of genetic data, virtually nothing is known about molecular interactions among members of the Arabidopsis MADS box transcription factor family. In Supplemental Table 3 online, an overview of the published interactions is given. Of 16 previously reported interactions, nine were also found in our study. The majority of the remaining seven interactions were only identified between truncated forms of the proteins, which provides a possible explanation why we did not detect them in our study with full-length proteins. We also used information on interactions between orthologous MADS domain proteins from other species. MADS factors are key regulators of plant development, and many of their important roles as developmental selector genes are conserved among various plant species, although it has also been suggested that diversification of

MADS activity after gene duplication may contribute to floral diversity (reviewed in Ferrario et al. (2004a)). In line with the evolutionary conservation of MADS box transcription factor

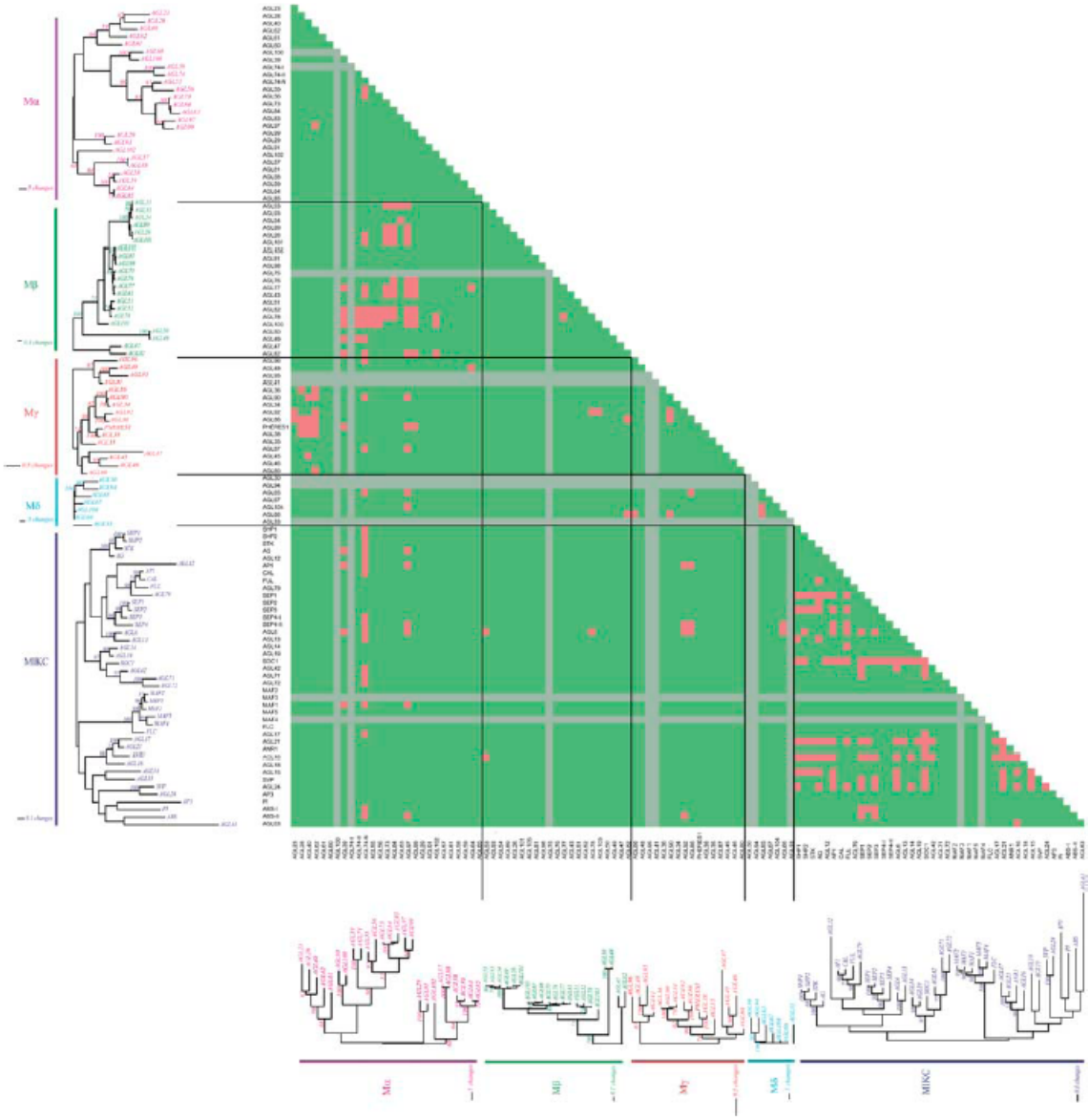


**Figure 1**. The Arabidopsis MADS Box Transcription Factor Interaction Matrix. The MADS box transcription factors are arranged according to their phylogenetic relationship as has been reported by Parenicová et al. (2003). The phylogenetic trees are indicated on the x and y axis with the different groups indicated (Ma, Mb, Mg, Md, and MIKC). Protein–protein interactions are represented by red blocks, no interactions by green blocks, and interactions that could not be tested by grey blocks.

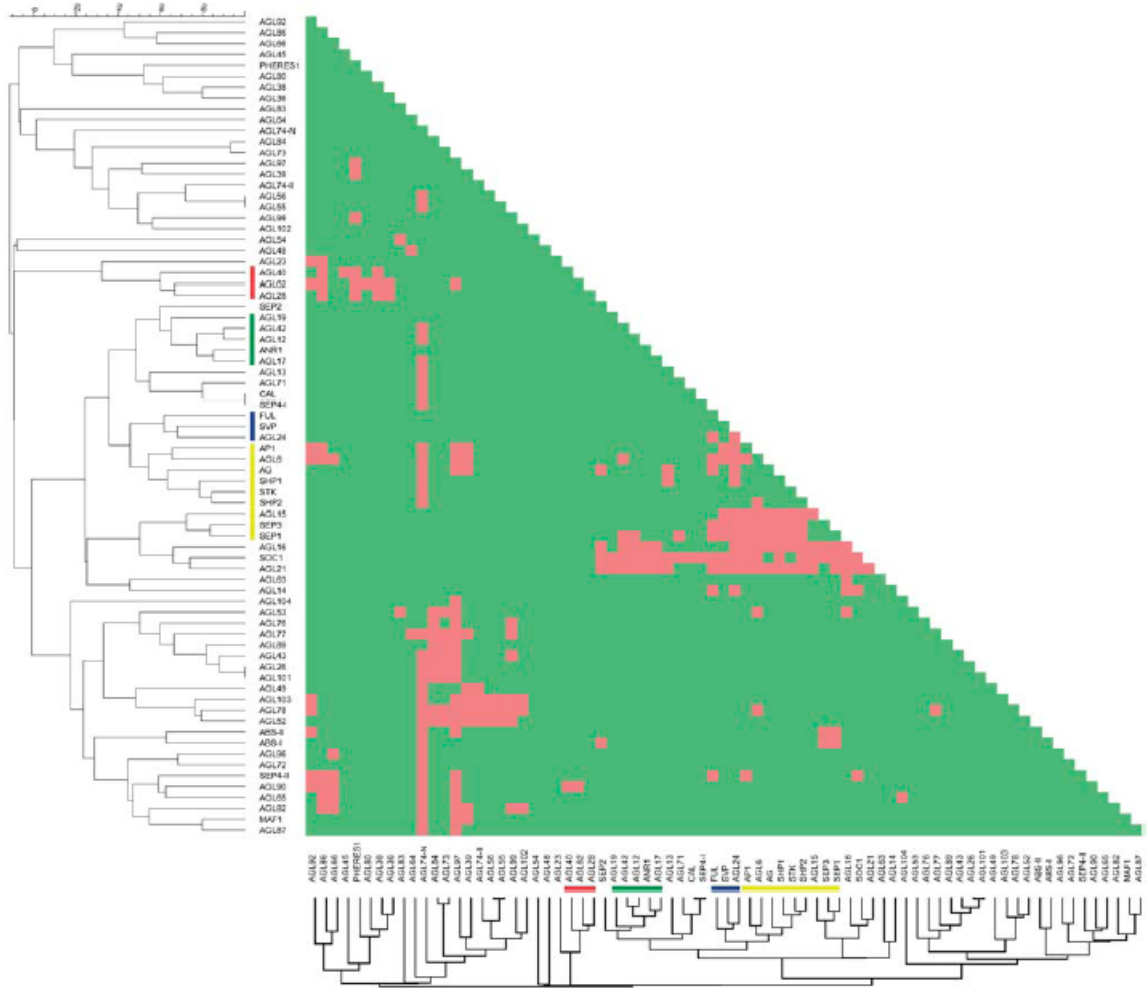

**Figure 2**. Interactome Map of the Arabidopsis MADS Box Transcription Factor Family. Proteins are organized based on hierarchical clustering of their protein–protein interaction patterns. Proteins that do not interact in the screen are omitted from this figure. Protein–protein interactions are indicated with red blocks and no interactions with green blocks. Presence of clustered proteins with a putative similar function is indicated with a colored bar on the left and bottom side of the figure: red for embryo, green for root, blue for flowering, and yellow for floral organs.

functions, the interaction patterns for specific MADS box proteins with identical functions, but from different species, have proven to be conserved (Favaro et al. 2002; Immink and Angenent 2002). To validate the data presented here, the literature was screened for putative interologs of the various Arabidopsis MADS box protein combinations. This analysis could be performed for the type II proteins only because no interaction with type I proteins have yet been reported for any plant species. Figure 3 shows the subset of Arabidopsis MADS box protein–protein interactions for which at least one homologous interaction has been found. An interaction between proteins

observed in a yeast two-hybrid assay can only be biologically relevant when they are present in the same cell and at the same moment. Hence, coexpression of the corresponding genes can be used for the validation of protein interaction data, even though the correlation of RNA and protein levels varies for different genes (Gygi et al. 1999; Beyer et al. 2004). We used the developmental data set of the AtGenExpress project (Schmid et al. 2005) (see Methods) to investigate whether there is a correlation between gene expression and protein interaction. In general, genes with similar functions, such as the ABC homeotic genes and the SEP genes (Pelaz et al. 2000) or the redundantly acting SHATTERPROOF1 (SHP1), SHP2, and SEEDSTICK (Pinyopich et al. 2003), genes clustered together (see Supplemental Figure 1 online). We asked more specifically how often genes are coexpressed in at least one sample using an absolute criterion for expression. This comparison revealed that almost 100% of the interacting proteins have an overlap in expression pattern of the corresponding genes, which is a prerequisite for a possible in planta interaction and relevance in Arabidopsis tissues (Figure 4). We next asked whether the expression patterns of interacting pairs were on average more similar than those of non-interacting pairs. Although the average Pearson correlation of expression levels of interacting genes was only slightly higher than of non-interacting genes, the distribution of non-interacting and interacting genes was significantly different. Specifically, the interacting pairs included a larger group of genes with more similar expression patterns (see Supplemental Figure 2 online), although there was also an excess of genes with contrasting expression patterns. A prominent case in this latter group was SHORT VEGETATIVE PHASE (SVP), a floral repressor (Hartmann et al. 2000) whose expression pattern is negatively correlated with those of SEP1, SEP3, and AP1, all of which play positive roles in flowering (Ferrario et al. 2004a).

**The Flower Induction and Flower organ Formation Sub-networks**

The regulation of flowering time is a complex process in which many environmental and internal signals are integrated, finally giving rise to a switch from vegetative to generative development at the appropriate time. MADS box transcription factors have shown to play pivotal roles in the flowering program and occupy many important positions in the hierarchical network (summarized and reviewed in Blázquez (2000); Simpson and Dean (2002)). Based on the interaction data obtained in this study, we tried to unravel two sub-networks composed of interactions between known MADS box proteins involved in flower induction and flower organ formation (Figure 5). The proteins AP1 and FRUITFULL

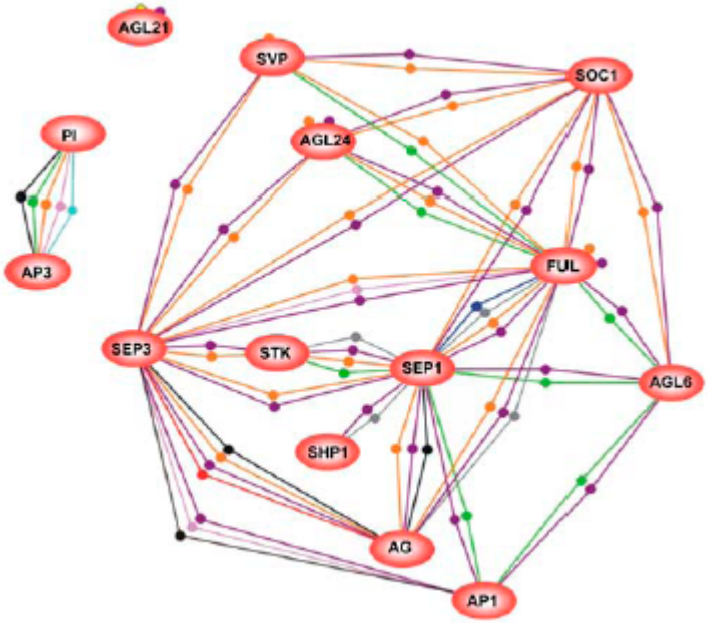


**Figure 3**. Subset of Arabidopsis MADS Box Transcription Factor Interactions Confirmed by Interologs.

(FUL) are present in both sub-networks, which would refer to their early and late function in flowering (Mandel et al. 1992; Ferrandiz et al. 2000). However, the most striking observation is that many of the floral organ identity proteins, such as AGAMOUS (AG), SEP1/2/3, and SHP1/2 proteins, interact not only with positive regulators of flowering, such as SUPPRESSOR OF CONSTANS 1 (SOC1) and AGAMOUS-LIKE 24 (AGL24), but also with a negative regulator, SVP, implying that there is both positive and negative crosstalk between the two pathways via protein interactions, as pointed out above.

## DISCUSSION

Interactions between proteins are essential for their activity and serve as the building blocks for the molecular networks that control biological processes in organisms. Here, we report a large protein–protein interaction study performed in plants, resulting in a near-complete interactome of Arabidopsis MADS domain transcription factors. Although derived from a heterologous system, these interaction patterns give valuable clues about the involvement of the MADS factors in certain processes. Some of the unexpected interactions, such as those between regulators of flowering time and floral pattern, may indicate the existence of hitherto unsuspected regulatory mechanisms. Duplication of MADS box genes appears to be a common phenomenon, not only giving rise to

functionally redundant genes, but also allowing diversification of developmental processes through changes in expression pattern or protein functions (reviewed in Smyth (2000); Ferrario et al. (2004a)). Completely redundant proteins are expected to have identical interaction patterns, and proteins playing a role in the same process are likely to have shared interaction partners. As expected, redundant proteins such as SEP1 and SEP3 (Pelaz et al. 2000) cluster together in the interaction matrix, as do members of the AG clade, which have partially overlapping functions (Favaro et al. 2003; Pinyopich et al. 2003). Similarly, proteins that may play a role in root development are grouped (AGL19, AGL42, AGL12, ANR1, and AGL17) (Rounsley et al. 1995; Zhang and Forde 1998; Alvarez-Buylla et al. 2000b; Burgeff et al. 2002), as are proteins known to be involved in the timing of flowering (e.g., SVP, AGL24, and FUL) (Ferrandiz et al. 2000; Hartmann et al. 2000; Yu et al. 2002; Michaels et al. 2003). The AGL6 protein has an interaction pattern closely resembling the AP1 interactions, suggesting that this protein plays a role in the flowering program as well. This hypothesis is strengthened by the fact that overexpression of OMADS1 from orchid (Oncidium Gower Ramsey), a gene closest in sequence to AGL6, resulted in extremely early flowering in Arabidopsis and loss of inflorescence indeterminacy (Hsu et al. 2003). Another protein for which the function recently has been elucidated by mutant analysis is AGL3 and based on its determined function has been renamed SEP4 (Ditta et al. 2004). Besides its function in floral organ formation, this protein appears to play a role

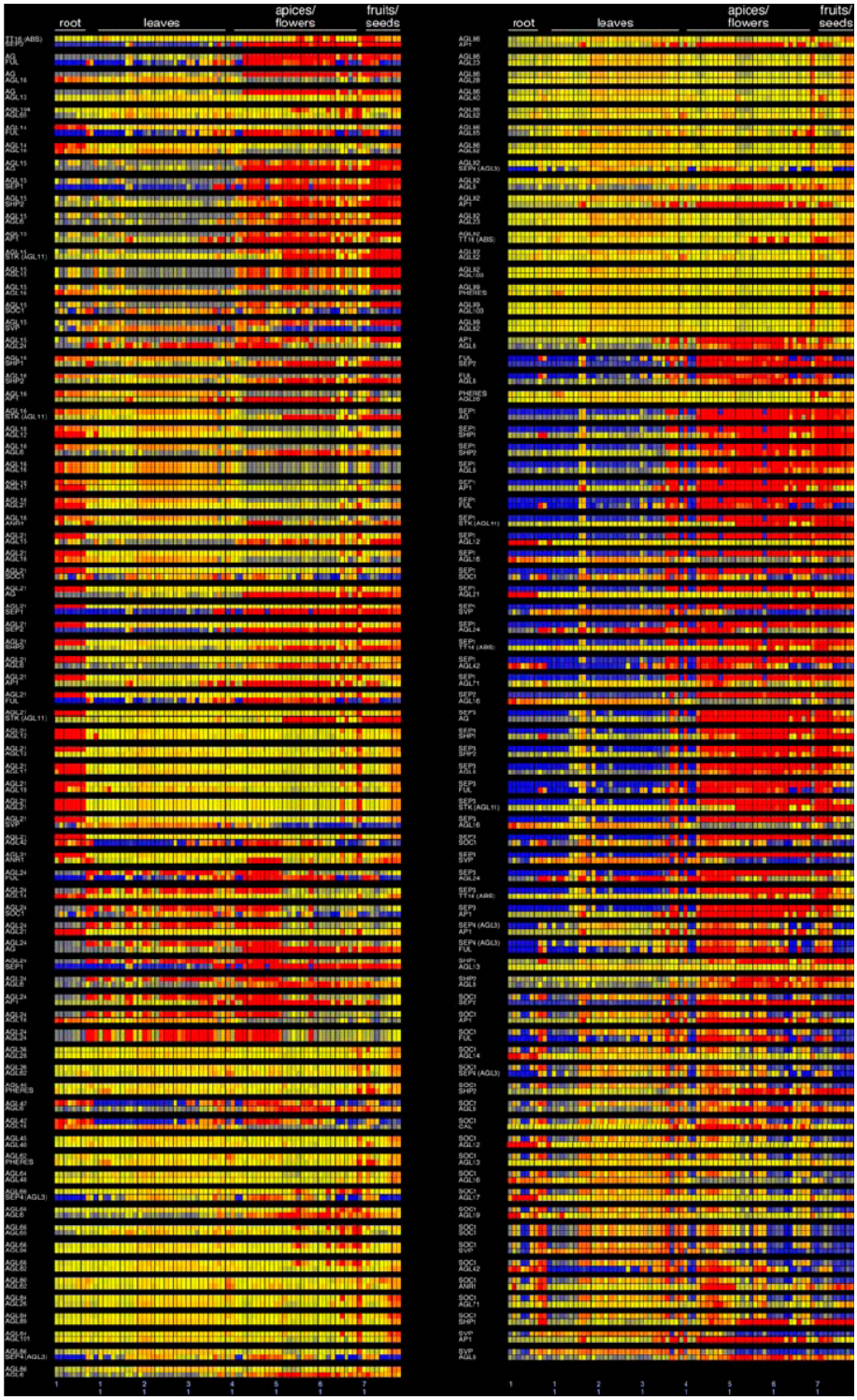


**Figure 4**. Comparison of Expression Patterns of Genes That Encode Interacting Proteins. The data from the AtGenExpress expression atlas are represented such that expression of each gene is normalized across the entire data set. The most important groups of tissues are indicated at the top and in detail numbered in the bottom (list of all tissues is presented in Supplemental Table 5 online). Blue indicates underexpression and red overexpression relative to the mean, with yellow expression levels that are close to the average for the corresponding gene.

in determining the floral meristem identity, redundantly with AP1 and CAULIFLOWER (CAL). Remarkably, SEP4 and CAL cluster together based on their interaction patterns, which also points to their redundant function. Interaction patterns may also provide clues about the role of the interacting proteins in a certain pathway, even when the majority of the proteins in the interaction cluster are unknown. An example is provided by the type I proteins, for which virtually no functional information is available. An exception is PHERES1 (PHE1; Köhler et al. (2003)), a target of the polycomb protein MEDEA that is involved in seed development (Grossniklaus et al. 1998). PHE1 interacts with AGL28, AGL40, and AGL62, which are all coexpressed with PHE1 in the embryo and cluster together according to their interacting patterns. This clearly points to their involvement in the same developmental process. Protein interactions that are clustered based on similar interaction patterns can serve as backbones for more complex molecular networks responsible for a particular function or pathway.

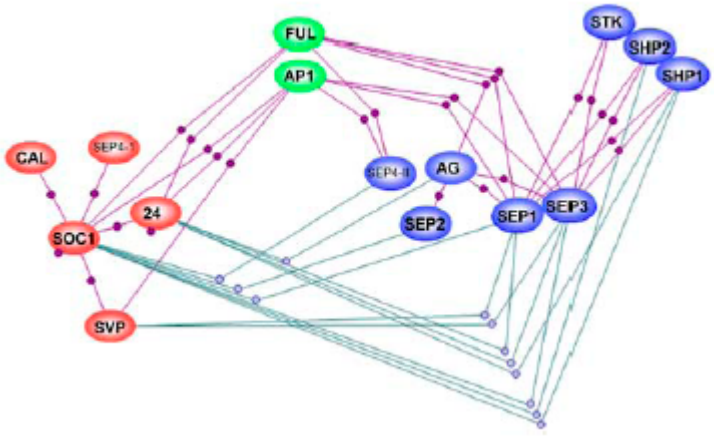


**Figure 5**. Representation of the Flower Induction and Flower Formation Networks. Proteins are indicated by ovals (red for the flower induction, blue for the flower formation network, green for the hubs), and interactions are represented by lines. The proteins SOC1 and AGL24 form a homodimer, which is indicated with a small dot next to the oval of the protein.

We have focused on two subnetworks, one for the flower induction and one for the flower organ formation pathway, which appear to be highly interconnected. Highly connected proteins can function as hubs to interconnect pathways that are either spatially or temporally separated. The proteins AP1 and FUL could serve as hubs between the flower induction pathway comprising interacting proteins such as SVP, SOC1, and AGL24, and the floral organ identity proteins. Both AP1 and FUL have a dual function in floral meristem identity (early function) and floral organ determination (late function) (Mandel et al. 1992; Ferrandiz et al. 2000), which is in line with the

fact that dimers are formed with both the flowering proteins and the floral homeotic proteins. Surprisingly, SVP, SOC1, and AGL24 also interact directly with the floral organ identity proteins. Both groups of genes share similar expression at the shoot apex, although the overlap on the cellular level is relatively limited. One possibility is that there is mutual negative feedback regulation, which would sharpen contrasting expression patterns (Heck et al. 1997; McKay and Cidlowski 1998). In such a scenario, the corresponding dimers would repress expression of both SVP and AP1/SEP1/SEP3, thus ensuring that overlap in expression pattern is minimized. An even more intriguing possibility is that there is overlap in expression pattern precisely at the moment when the shoot apical meristem is transformed into a generative meristem. Then, these dimers could serve not only as repressors of the early flowering genes, but also as activators of the floral organ identity genes, further sharpening the transition to flowering. Positive autoregulatory feedback loops have been reported for the class B homeotic genes in Arabidopsis (Goto and Meyerowitz 1994; Samach et al. 1997) and Antirrhinum (Schwarz-Sommer et al. 1992; Tröbner et al. 1992) and more recently for AG in Arabidopsis (Gómez-Mena et al. 2005). A prerequisite for the negative autoregulatory feedback loop theory presented here is that the potentially repressed genes contain the motif for MADS box protein binding, the so-called CArG-box (Shore and Sharrocks 1995). All three genes, SVP, SOC1, and AGL24, contain a perfect CArG-box [CC(A/T)6GG] in their putative regulatory sequences, which is, for example, lacking in the CAL gene for which the gene product did not reveal interactions with the floral identity proteins. Further analyses are required to provide evidence for these negative feedback loops, which could facilitate the major switches in meristem identity. However, first indications for this theory are already available from genetic data. The SVP and AGL24 proteins, which are very close in sequence and have similar interaction patterns, have an opposite effect on flowering time (Hartmann et al. 2000; Yu et al. 2002; Michaels et al. 2003). This suggests that SVP and AGL24 are acting at the molecular level as floral repressor and inducer, respectively, by dimerization with the same partners. In recent studies, constitutive expression of either SVP or AGL24 resulted, as expected, in late and early flowering, respectively (Masiero et al. 2004; Yu et al. 2004). However, in contradiction with the opposite flowering time phenotypes, these transgenic Arabidopsis plants revealed similar alterations in the flower. The flowers have features of ap1 mutant flowers and often contain greenish sepaloid petals and showed indeterminacy. These observations are in accordance with the proposed model that the flowering time proteins are normally switched off in the flower by negative feedback mechanisms, which are controlled by heterodimers containing both flowering time and floral organ identity proteins. In

case of ectopic expression using the strong constitutive 35S promoter of Cauliflower mosaic virus, the negative feedback loops are overruled, giving rise to floral mutations. The altered floral phenotypes from 35S:SVP and 35S:AGL24 plants can be explained by our observed protein interactions. Both SVP and AGL24 form interactions with the floral organ identity proteins, such as AP1, AG, and SEP3. In the overexpressers, these protein complexes may act in a dominant-negative manner on the floral organ identity proteins. Similar floral defects were obtained upon overexpression of SOC1, which functions as an accelerator of flowering (Borner et al. 2000; Lee et al. 2000; Samach et al. 2000). Detailed analyses with the petunia UNSHAVEN protein, the putative functional homolog of SOC1, has also shown that in this case the floral phenotype is obtained by a dominant negative effect on the floral organ identity proteins (Ferrario et al. 2004b). In summary, all results from mutant and overexpression analyses and the interaction data presented here for SVP, AGL24, and SOC1 give strong indications for the proposed negative feedback loop model. The results presented here provide a glimpse of the complex interaction network for the Arabidopsis MADS domain transcription factor family. The current available protein interaction map still represents a largely static view of the cellular processes regulated by the interactome. Technologies such as fluorescence resonance energy transfer (Immink et al. 2002) and bimolecular fluorescence complementation (Bracha-Drori et al. 2004; Walter et al. 2004) are powerful tools for in vivo studies aimed at analyzing the dynamics of changing protein interactions. Unraveling the dynamic spatial and temporal changes in binary and macromolecular assemblies and the de novo complex assembly in response to varying external stimuli will provide a detailed understanding of biological systems.

## METHODS

### Cloning of the Full-Length MADS Box Transcription Factors

A detailed description of the amplification of the open reading frames and subsequent cloning in yeast two-hybrid vectors is given in Supplemental Text 1 online. In summary, 102 open reading frames were cloned: 99 in the bait vector and 102 in the prey vector.

**Yeast Two-Hybrid Analysis**

The bait vectors were transformed into yeast strain PJ69-4a (MATa; James et al. (1996)) and all prey vectors into strain PJ69-4a (MATa; James et al. (1996)) and selected on SD plates lacking Leu and Trp, respectively. Subsequently, overnight cultures were grown (308C, 300 rpm) from single colonies of each transformant in selective SD medium and systematically mated with each other by spotting 5-mL droplets of the liquid cultures on top of each other on SD complete plates (Nunc Omnitray; VWR International, Amsterdam, The Netherlands) containing all the essential amino acids. The spotting was performed in a systematic manner in a grid of 96 spots/plate by a pipetting robot (Genesis RSP150 workstation; Tecan, Maennedorf, Switzerland). In addition, some negative control combinations were spotted, for which water was used instead of either a bait or prey culture. Subsequently, the plates were incubated at 308C for 16 h, and afterwards the yeast was transferred to SD plates lacking both Leu and Trp with disposable 96-pin replicators (Nunc-TSP; VWR International) to select for diploid yeast containing both plasmids. After 2 d of growth at 30°C, the yeast was transferred to two different selection plates containing SD medium lacking Leu, Trp, and Ade and SD lacking Leu, Trp, and His, supplemented with 5 mM 3-amino-1,2,4-triazole. These plates were incubated at 20°C and scored for growth of yeast and hence protein–protein interaction events after 5 d. The screening was performed in duplicate, yielding in theory eight data points for each combination, four times with protein A as bait and B as prey (two scores from the Ade selection and two scores from the His selection) and four times reciprocally, with protein B as bait and A as prey. In case of autoactivation for one of the two proteins, just four data points were obtained for the specific combination. The mating efficiency appeared to be 100%, and where water was used for mating, either instead of a bait culture or instead of a prey culture, no growth was obtained on medium selecting for the presence of the two plasmids or on the media selecting for interactions (see Supplemental Figure 3 online). This shows that no cross-contamination occurred as a result of the procedure that followed. A combination was scored as a true interaction when it resulted in growth for at least one of the two selection markers in both screenings, but almost all positively scored combinations grew on both selection media.

**Data Analysis**

All protein–protein interaction data were transferred to Microsoft Excel sheets (Redmond, WA), and for easier data analyses, the interaction data was made reciprocal. One data matrix was made with all MADS box proteins, Matrix1, and one matrix with MADS box proteins that had at least

one protein–protein interaction, Matrix2. Both matrixes were subjected to GeneMaths software (Applied Maths BVBA, Sint-Martens- Latem, Belgium) for further data analyses. Matrix1 was organized based on the phylogenetic distribution of all Arabidopsis thaliana MADS box proteins according to Parenicová et al. (2003). Cluster analysis was performed on Matrix2 with Pearson correlation coefficient and UPGMA algorithm on both the rows and columns. In both cases, the data are represented in one direction (not reciprocal).

**Coexpression Analysis**

The developmental set of the AtGenExpress expression atlas (ftp:// ftp.arabidopsis.org/home/tair/Microarrays/Datasets/AtGenExpress/; http://weigelworld.org/resources/microarray/AtGenExpress) (Schmid et al. 2005) was analyzed for expression of MADS box genes. Expression estimates were obtained using gcRMA (http://bioconductor.org), a modification of the robust multiarray analysis algorithm (Irizarry et al. 2003). A threshold of log2 *$* 3 was applied to identify overlap in tissues with expression of genes. Approximately75% of the Arabidopsis MADS family is represented on the Affymetrix GeneChip ATH1 (Santa Clara, CA).

**ACKNOWLEDGMENTS**

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# Chapter 3

# MADS-box protein complexes controlling carpel and ovule development in Arabidopsis

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**ABSTRACT**

The *AGAMOUS* (*AG*) gene is necessary for stamen and carpel development and is part of a monophyletic clade of MADS-box genes that also includes *SHATTERPROOF 1*(*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*). Here, we show that ectopic expression of either the *STK* or *SHP* gene is sufficient to induce the transformation of sepals into carpeloid organs bearing ovules. Moreover, the fact that these organ transformations occur when the *STK* gene is expressed ectopically in *ag* mutants shows that *STK* can promote carpel development in the absence of *AG* activity. We also show that STK, AG, SHP1, and SHP2 can form multimeric complexes and that these interactions require the SEPALLATA (SEP) MADS-box proteins. We provide genetic evidence for this role of the SEP proteins by showing that a reduction in SEP activity leads to the loss of normal ovule development, similar to what occurs in *stk shp1 shp2* triple mutants. Together, these results indicate that the SEP proteins, which are known to form multimeric complexes in the control of flower organ identity, also form complexes to control normal ovule development.

**INTRODUCTION**

In angiosperms, the ovule is located within the pistil, which consists of one or several fused carpels. The unfertilized mature ovule is formed by a haploid embryo sac surrounded by one or two integuments. The ovule is connected to the maternal body through the funiculus. Several important events occur during ovule development: the ovule primordium has to be formed, ovule identity has to be specified, followed by pattern formation and morphogenesis. In recent years, the ovule, particularly of Arabidopsis, has emerged as a model system in which to study the genetic and molecular bases of organogenesis (Grossniklaus and Schneitz 1998). Several genes have been identified that are involved in the initiation and development of structures as integuments or the gametophyte. In Arabidopsis, ovules develop inside two fused carpels. *AGAMOUS* (*AG*) plays a primary role in specifying carpel formation, because *ag* mutants completely lack carpels (Yanofsky et al. 1990). However, when the *ag* mutant is combined with mutant alleles of the *APETALA2* (*AP2*) gene, an ERF-type transcription factor, ectopic carpeloid structures, including ovules, are observed on the margins of the sepals (Bowman et al. 1991a), indicating that carpeloid features can develop in the absence of *A* activity. When the *ag ap2* mutant was combined with mutant alleles of *SHATTERPROOF1* (*SHP1*) and *SHP2*, forming the *ag ap2 shp1 shp2* quadruple mutant, all

carpeloid features, including ovules, were absent on the first-whorl organs (Pinyopich et al. 2003), showing that *SHP1* and *SHP2* are required for *AG*-independent carpel and ovule development. *shp1* and *shp2* single mutants do not exhibit any phenotypic effect, and the double mutant by itself does not affect carpel identity, because the *shp1 shp2* double mutant is disturbed only in dehiscence zone formation in the fruit, by which these mutant fruit are unable to shatter their seeds (Liljegren et al. 2000).

Interestingly, it was shown recently that *SHP1* and *SHP2* act redundantly with *SEEDSTICK* (*STK*; previously *AGL11*) in promoting ovule identity (Pinyopich et al. 2003). In the *stk shp1 shp2* triple mutant, normal ovule and seed development was disrupted completely, with some of the ovules converted to leaf-like or carpel-like structures. The homeotic transformation of ovules into carpeloid structures was shown previously in petunia as a result of the co-suppression of two MADS-box genes, *FLORAL BINDING PROTEIN 7* (*FBP7*) and *FBP11*, which are homologous with *STK* (Angenent et al. 1995). Besides its role in ovule development, *STK* also is required for normal funiculus development and seed dispersal (Pinyopich et al. 2003).Several studies have shown that MADS-box transcription factors act by the formation of multimeric complexes. The first example was reported for the *Antirrhinum majus* MADS-box transcription factors SQUAMOSA (SQUA), DEFICIENS (DEF), and GLOBOSA (GLO). Genetic evidence was obtained that *SQUA* together with *DEF* and *GLO* are needed for the establishment and maintenance of whorled phyllotaxy. The genetic interactions between SQUA, DEF, and GLO were confirmed at the molecular level by showing that the three MADS-box proteins forma ternary complex on the DNA (Egea-Cortines et al. 1999). The formation of multimeric MADS-box protein complexes that promote flower organ development also was shown in Arabidopsis. Genetic studies showed that the closely related and functionally redundant MADS-box genes SEPALLATA1 (SEP1), SEP2, and SEP3 were necessary to determine the identity of petals, stamens, and carpels (Pelaz et al. 2000). Single sep mutants showed only subtle phenotypes, whereas the triple mutant produced indeterminate flowers composed of only sepals. This phenotype is strikingly similar to that of bc (ap3 ag or pi ag) double mutants, indicating that the SEP genes are in some way required for the activity of the class-B and -C organ identity genes. Experiments performed by Honma and Goto (2001) gave the molecular clue to the observed genetic interactions. They showed that in yeast and in vitro, the SEP3 protein establishes the interaction between AP1 and PISTILLATA (PI)/AP3 proteins and between AP3/PI and AG. Furthermore, overexpression studies in quadruple transgenic Arabidopsis plants overexpressing AP1-PI-AP3-SEP3 showed that vegetative leaves were

converted to petal-like organs and that plants overexpressing PI-AP3-SEP3-AG had vegetative leaves converted to stamen-like organs. These data provided evidence that tetrameric MADS-box transcription factor complexes determine the identity of the floral organs, at least in petals and stamens. Here, we report that STK, SHP1, and SHP2 overexpression in Arabidopsis results in homeotic conversions of sepals to carpeloid structures, including ovules. Interestingly, carpel formation also was observed when STK was overexpressed in the ag mutant, probably as a result of the ectopic expression of SHP1 and SHP2 induced by STK, indicating that AG activity is not needed to induce these homeotic transformations. This finding confirms that SHP1 and SHP2 are sufficient to promote carpel identity in the absence of AG. To investigate whether the redundancy between STK, SHP1, and SHP2 in promoting ovule identity and that between AG, SHP1, and SHP2 in promoting carpel identity is based on a biochemical interaction, we performed yeast two- and three-hybrid experiments. These experiments showed that STK cannot interact directly with AG, SHP1, and SHP2, although an interaction was obtained with SEP proteins. Genetic evidence for the role of SEP proteins in the formation of an ovule identity-promoting complex came from analysis of the SEP1/sep1 sep2 sep3 mutant, in which a large number of ovules lose their identity and are transformed into carpel- and leaf-like structures.

## RESULTS

### Ectopic Expression of *STK* in Transgenic Arabidopsis Plants Resulted in Ectopic Carpel and Ovule Formation

Previous experiments showed that the redundant petunia genes *FBP7* and *FBP11* were necessary for ovule identity determination and sufficient to induce ectopic ovule formation on sepals and petals (Colombo et al. 1995). *STK* is highly similar to these petunia genes and, together with *SHP1* and *SHP2*, essential for ovule formation (Pinyopich et al. 2003). To determine whether ectopic *STK* expression also is sufficient to induce ectopic ovule formation, Arabidopsis plants were transformed with a chimeric gene construct in which the *STK* cDNA coding region was fused to the Cauliflower mosaic virus(CaMV) 35S double enhancer containing promoter (Benfey et al. 1990a, b). Sixty independent transgenic Arabidopsis lines were selected. Expression analysis of the transgene by reverse transcriptase–mediated (RT) PCR using RNA extracted from leaves revealed that of all the transgenic lines expressed STK ectopically (data not shown). Forty-five transgenic plants flowered extremely early (before the fourth leaf appeared), and the flowers of these plants had petals and

stamens that were reduced in size; sometimes the petals were completely absent (Figure 1B).Furthermore, the first-whorl sepals were converted to carpeloid organs on which ovules developed, and in a few cases, the bract leaves were homeotically converted to carpeloid organs on which ovules developed (Figures 1C and 1D). To study these homeotic changes in more detail, scanning electron microscopy analysis was performed. As shown in Figures 1F and 1G, stigmatic papillae developed on the edges of the first-whorl organs. The carpeloid organs in the first whorl very often were folded, and on the inner site, ovule-like structures developed (Figure 1H). Structures that are typical for wild-type ovules, such as the developing inner and outer integuments, the funiculus, and the nucellus, could be recognized clearly in these ectopic ovules (Figures 1I and 1J). RT-PCR performed on leaves of the CaMV 35S::STK transgenic plants showed that ectopic STK expression resulted in the induction of ectopic SHP1, SHP2, AG, and SEP3 expression (Figure 2). Ectopic expression of SHP1 and SHP2 (data not shown) resulted in the conversion of sepals to carpeloid structures, as described for STK, and the conversion of petals to staminoid structures, confirming the data published by Liljegren et al. (2000). These experiments show clearly that ectopic STK, SHP1, and SHP2 activities are able to induce the carpel and ovule pathways in these transgenic plants.

**Ectopic Expression of *STK* in the *ag-3* Mutant**

The carpeloid structures observed in the first-whorl floral organs of the *STK*, *SHP1*, and *SHP2* overexpression plants resembled closely those observed in Arabidopsis plants in which *AG* was expressed ectopically (Mizukami and Ma 1992). Furthermore, RT-PCR revealed that *STK*, when expressed ectopically, induced ectopic *AG* expression. To understand whether the observed homeotic conversions were dependent on *AG* activity, we transformed *ag-3* mutant plants (Bowman et al. 1989; Bowman et al. 1991a) with the construct for ectopic *STK* expression. In the *ag-3* mutant flowers petals developed in place of stamens, and instead of carpels, four sepals arose that constituted the outer whorl of another inner *ag* flower (Figures 3A and 3B). The CaMV 35S::*STK* construct was used to transform *ag-3*/_ heterozygous plants, which were distinguished from wild-type plants using PCR primer-introduced restriction analysis (Jacobson and Moskovits 1991). We analyzed 80 transgenic CaMV 35S::*STK* plants segregating for the *ag* mutant allele, all of which ectopically expressed *STK*. Forty heterozygous plants (*ag-3*/_) ectopically expressing *STK* showed the same phenotype as those observed previously when wild-type plants were transformed: they were small as a result of extremely early flowering, and their sepals frequently were homeotically

converted to carpeloid organs. Twenty transgenic *ag* mutant (*ag-3/ag-3*) plants ectopically expressing *STK* were obtained and analyzed. These mutants flowered extremely early as well, maintaining a small size, and generally the flowers resembled typical *ag-3* flowers. However, in two plants, homeotic conversion of the first-whorl sepals to carpeloid organs was observed (Figures 3C to 3E), suggesting that *AG* activity is not needed for the *STK*-induced homeotic transformations.

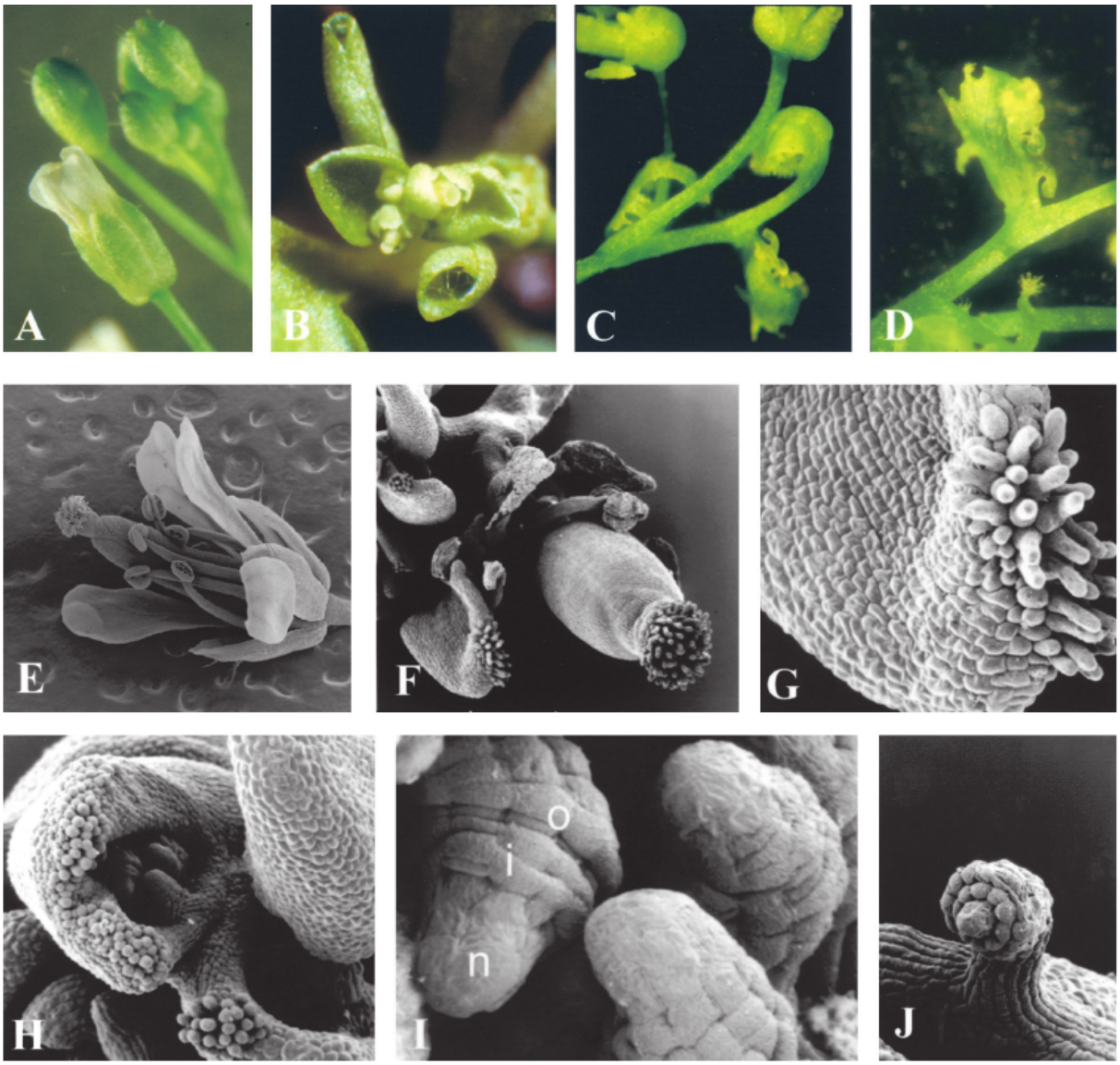


**Figure 1.** Flower Morphology of CaMV 35S::STK Transgenic Arabidopsis Plants.

Stereomicroscopic images are shown in **(A)** to **(D)**, and scanning electron microscopy images are shown in **(E)** to **(J)**.

**(A)** Wild-type flower.

**(B)** Inflorescence of a CaMV 35S::STK transgenic plant.

**(C)** and **(D)** Carpeloid bracts with ovules of a CaMV 35S::STK transgenic plant.

**(E)** Wild-type flower.

**Figure 1** (continued)**: (F)** Flower of a CaMV 35S::STK transgenic plant.

**(G)** Carpeloid sepal with stigmatic tissue.

**(H)** Curled carpeloid sepal with ectopic ovules.

**(I)** and **(J)** Ectopic ovules, with outer (o) and inner (i) integuments and nucellus (n).

Furthermore, in a flower of one of these plants, a pistil-like structure developed in the fourth whorl (Figure 3F). However, when we opened this pistil, no ovules were detected. RT-PCR analyses performed on leaves of the transgenic plants that showed homeotic conversions (Figure 2) revealed that *STK* induced the ectopic expression of *SHP1*, *SHP2*, and *SEP3*, suggesting that the observed phenotypes could be promoted by *SHP1* and *SHP2* genes that are responsible for *AG*-independent carpel development (Pinyopich et al. 2003). Surprisingly, ovules were detected on none of the first-whorl organs that showed carpeloid features.

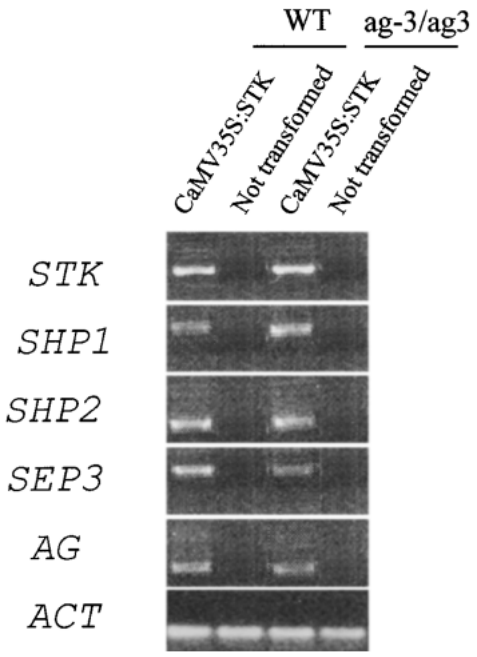


**Figure 2.** Expression Analyses by RT-PCR of Genes Induced in Arabidopsis Wild-Type and ag-3 Mutant Plants Ectopically Expressing *STK*. RT-PCR analysis using RNA extracted from bract leaves of wild-type (WT) and *ag-3* mutant plants (*ag/ag*) in which *STK* was expressed ectopically (35S::STK). As a control, this analysis also was performed on plants that were not transformed. RT-PCR was performed using independent transformants, all of which gave similar results. Representative RT-PCR results are shown. In the CaMV 35S::STK plants, the *SHP1*, *SHP2*, *SEP3*, and *AG* genes all were induced. In the *ag-3* mutant, the induced *AG* RNA encoded a nonfunctional transcript. ACT, control amplification on actin.

## STK Interacts with AG in a Multimeric Complex

To investigate whether *STK*, *SHP1*, *SHP2*, and *AG* redundancy in promoting ovule and carpel

formation was based on a biochemical interaction, we performed GAL4-based yeast two-hybrid experiments to assay the interactions between STK, AG, SHP1, and SHP2. The coding parts of the *AG*, *SHP1*, *SHP2*, and *STK* cDNAs were fused to the activation domain (AD) and binding domain (BD) and tested for interaction. In this assay, STK was not able to interact with AG, SHP1, and SHP2, and no interactions between SHP1, SHP2, and AG were observed (Figure 4A). Furthermore, none of the proteins was able to form homodimers. Because previous studies showed that the SEP proteins interact with AP1, AP3, PI, and AG in the control of organ identity (Honma and Goto 2001; Pelaz et al. 2001b), we tested whether or not the SEP proteins could interact similarly with STK. These assays showed that SEP3 interacted with STK (Figure 4A). Because STK, SEP3, and AG all are expressed during ovule development, we wondered whether the STK and AG proteins, which do not appear to interact on their own, might form a multimeric complex that also includes SEP3. To test this notion, yeast three-hybrid experiments were performed by fusing the SEP3 protein with the nuclear localization signal of the TFT vector (Egea-Cortines et al. 1999). As presented in Figure 3B, yeast strain PJ69-4A was able to grow on selective medium only when all three proteins were expressed, showing that an interaction between STK and AG can be mediated by SEP3. Furthermore, as shown in Figure 4A, SHP1 and SHP2 also were able to form heterodimers with SEP3. Therefore, we tested SEP3 and combinations of SHP1, SHP2, STK, and AG in the three-hybrid system. As shown in Figure 4B, SHP1 and SHP2 interacted, via SEP3, strongly with AG and weakly with STK. In fact, all combinations could be made using SEP3 as an intermediate, even interactions with themselves.

### *SEP* Genes Are Required for Ovule Development

The data obtained in the yeast assays described above suggest that SEP proteins are necessary to form transcription factor complexes that control ovule development. However, previous studies have failed to reveal a role for the *SEP* genes during ovule development, because the *sep* triple mutant completely lacks carpels. To reveal genetic evidence that the *SEP* genes are involved in ovule development, we reduced *SEP* activity by examining *SEP1/sep1 sep2 sep3* mutant plants. Interestingly, the ovules of these plants (Figure 5B) have a dramatic phenotype that closely resembles that observed in the *stk shp1 shp2* mutant (Figure 5C). Normal ovule and seed development was disrupted, and some of the ovules were converted to leaf-like or carpel-like structures. These data demonstrate that the *SEP* genes are required for normal ovule development.

## DISCUSSION

### Ectopic Expression of *STK*, *SHP1*, and *SHP2* Induce Carpel and Ovule Development in Arabidopsis

Ovule development is a complex process that can be divided into distinct phases, such as organ identity determination, pattern formation, and morphogenesis (Schneitz K. 1995). A large number of Arabidopsis mutants have been identified that display altered ovule development. The analysis of these mutants and the cloning of their corresponding genes have started to provide some insight into the processes that control ovule morphogenesis. Until recently, the Arabidopsis genes that control ovule identity determination were not identified. The only information on this early phase in ovule development came from studies in petunia, in which two redundant MADS-box genes, *FBP7* and *FBP11*, which specify ovule identity, were identified (Angenent et al. 1995; Colombo et al. 1995). Cosuppression of both genes resulted in the homeotic conversion of ovules to carpeloid structures, and ectopic expression of these genes induced ectopic ovule formation on sepals and petals. Recently, it was shown that in Arabidopsis, ovule identity is promoted by three redundant genes: *SHP1*, *SHP2*, and *STK* (Pinyopich et al. 2003). In the *shp1 shp2 stk* triple mutant, all ovules are disturbed in their development, and some of them are homeotically transformed into carpeloid organs.

Furthermore, it has been shown that an increased number of ectopic ovules were converted to carpeloid organs in the *ag ap2* double mutant with respect to the *ap2* single mutant (15%), indicating that *AG* also promotes ovule identity (Western and Haughn 1999). To determine whether *STK*, *SHP1*, and *SHP2*, like *FBP7* and *FBP11*, are able to induce ectopic ovule formation, we ectopically expressed these genes in Arabidopsis. These experiments showed that all of these genes induce homeotic conversions of sepals to carpeloid organs on which ovules developed. This result is similar to that obtained in petunia.

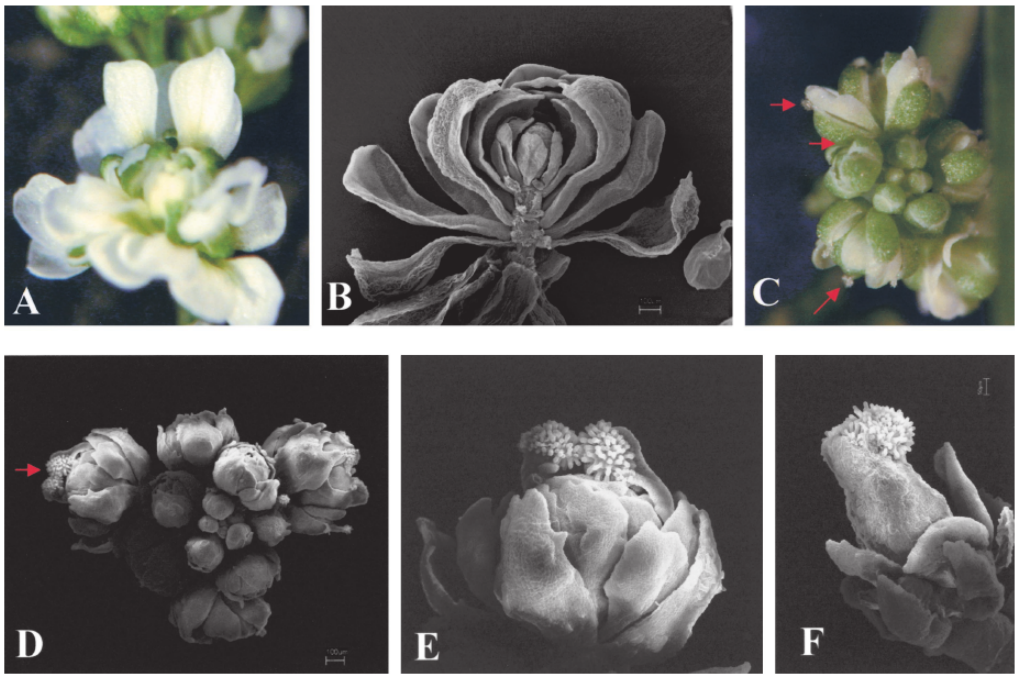


**Figure 3**. Ectopic Expression of *STK* in the *ag-3* Mutant. Stereomicroscopic images are shown in **(A)** and **(C)**, and scanning electron microscopy images are shown in **(B)** and **(D)** to **(F).**

**(A)** and **(B)** *ag-3* mutant flower.

**(C)** and **(D)** Inflorescence of a transgenic ag-3 mutant plant ectopically expressing *STK*. Stigmatic tissues on top of the carpeloid sepals are indicated by the arrows.

**(E)** Close-up of a single flower of the inflorescence shown in (D). Stigmatic papillae are clearly visible.

**(F)** Flower of a transgenic *ag-3* mutant plant ectopically expressing *STK*. A pistil-like structure develops in this flower.

However, in petunia, homeotic conversions were restricted to the change of the sepal inner epidermis to placental cells on which ovules developed. No other pistil features were observed in the first whorl organs of these transgenic petunia plants. Furthermore, occasionally, ovules also developed on the petals, without the development of any other detectable carpel features. This finding led to the conclusion that, at least in petunia, these two MADS-box genes were sufficient to induce ovule development without the presence of carpel structures. Although this might be true, molecular analysis showed that in the sepals and petals of these transgenic petunia plants, the class-C genes *FBP6* and *pMADS3* are induced (Colombo et al. 1995). That the induction of these genes did not result in severe homeotic conversions of sepals to carpeloid organs is not surprising, because the overexpression of *FBP6* does not have any effect on sepals, and ectopic *pMADS3* expression had only a very mild effect on sepals (Tsuchimoto et al. 1993; Kater et al. 1998). Our experiments with Arabidopsis show that ectopic ovule formation always is linked to the development of carpeloid structures. *STK*, which is expressed specifically in the ovules, promotes

ovule identity but is not a carpel identity gene, in contrast to *SHP1* and *SHP2*, both of which are able to promote carpel and ovule identity (Pinyopich et al. 2003). The carpel structures that develop on the first-whorl organs of transgenic Arabidopsis plants that ectopically express *STK* are largely the result of the induction of *AG*. In the *ag-3* mutant, ectopic expression of *STK* very rarely induced carpel formation in the first-whorl organs, indicating that *AG* is most efficient in inducing carpel formation. We never observed ovules on the carpeloid structures that developed in the *ag-3* mutant overexpressing *STK*. This finding could be attributable to the fact that the most effective complex that promotes ovule identity contains AG (e.g., a complex consisting of AG-SEP3-STK). Our yeast three-hybrid assays are in agreement with this finding, because the stronger interactions were obtained when AG was part of the complex.

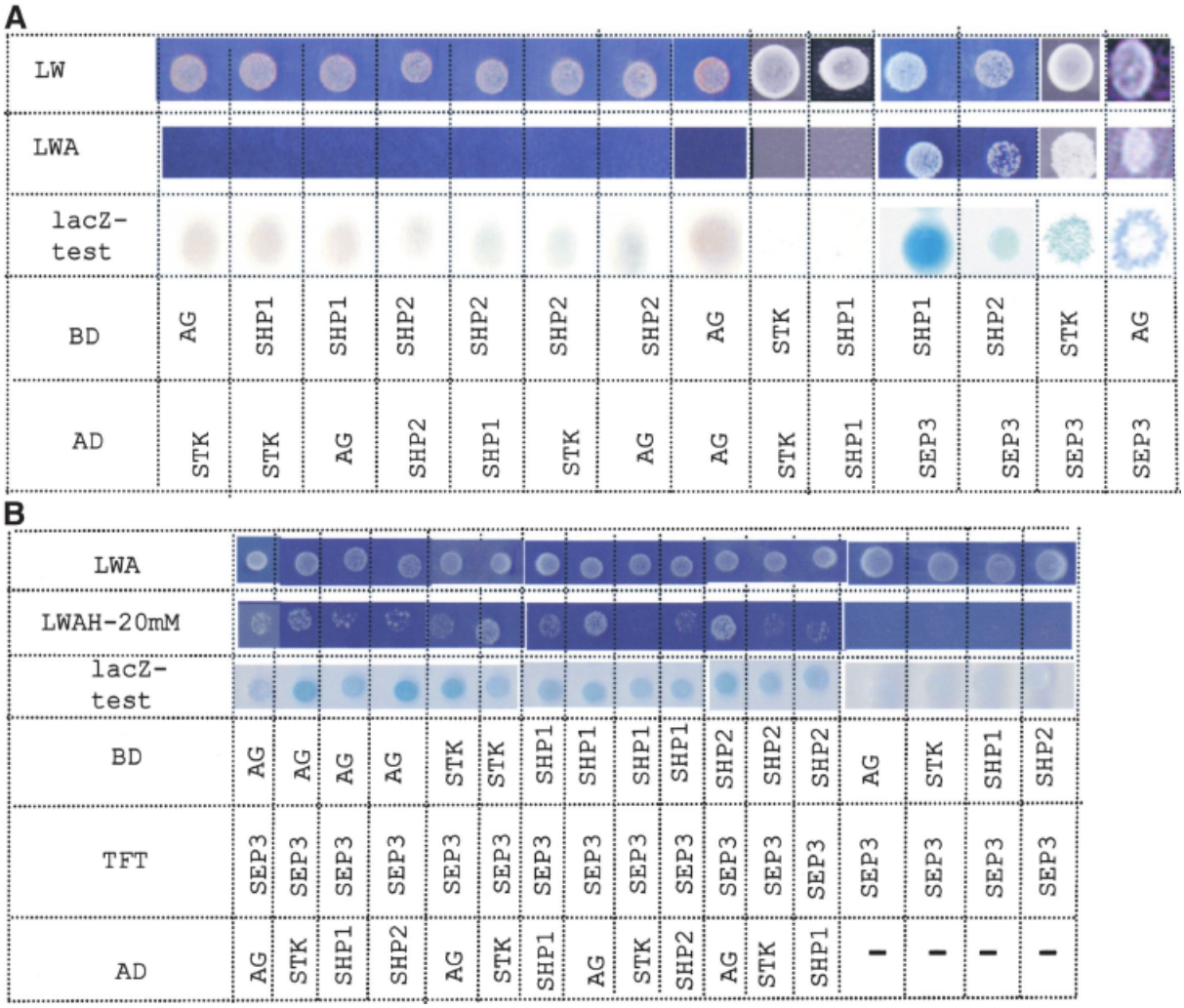


**Figure 4.** Interactions between MADS-Box Factors Detected by Yeast Two- and Three-Hybrid Assays.

**(A)** Yeast two-hybrid assays. MADS-box coding sequences were cloned in the pBD and pAD vectors. Yeast PJ69a was transformed with combinations of pBD and pAD constructs, and the presence of both vectors was confirmed by growth on dropout medium lacking Leu and Trp (LW). Interactions were assayed on selective dropout medium lacking Leu,

**Figure 4** (continued): Trp, and adenine (LWA) and by a -galactosidase (lacZ) assay.
**(B)** Yeast three-hybrid assays. To determine whether SEP3 could bridge the interaction between STK, AG, SHP1, and SHP2, yeast three-hybrid as-
says were performed. SEP3 was cloned in the pTFT1 vector and transformed to yeast together with pAD and pBD vectors containing different combinations of MADS-box coding **Figure 4** (continued) sequences. The presence of the three constructs was tested by plating on dropout medium lacking Leu, Trp, and adenine (LWA). Interactions were tested on dropout medium lacking Leu, Trp, adenine, and His (LWAH) with different concentrations of 3-aminotriazole.
Only the results with 20 mM 3-aminotriazole are shown. Furthermore, interactions were tested by -galactosidase (lacZ) assays.

## MADS-Box Proteins Form Complexes to Promote Ovule and Carpel Identity

To determine whether STK interacts with the carpel and ovule identity-promoting proteins AG, SHP1, and SHP2, yeast two-hybrid experiments were performed. These experiments revealed no interaction. Because it has been shown that SEP3 can mediate the interaction between AG and the class-B protein dimer AP3-PI (Honma and Goto 2001), we tested STK, SHP1, and SHP2 for an interaction with SEP3. These experiments confirmed that SEP3 is able to interact with all of them. Subsequently, yeast three-hybrid experiments showed that ternary complexes can be formed using all possible combinations of SHP1, SHP2, STK, and AG as long as SEP3 is added as a mediator. Evaluation of the strength of the interactions in the yeast assays by observing growth on dropout medium with different concentrations of 3-aminotriazole and analyzing the lacZ color assay (data not shown) indicated that the interaction between AG, SEP3, and STK or AG, SEP3, and one of the SHP proteins was strong. On the other hand, the interaction (mediated by SEP3) between STK and SHP proteins and interactions such as STK-SEP3-STK, SHP-SEP3-SHP, and AG-SEP3-AG all were weaker. The observed biochemical interactions could explain the observed redundancy between SHP1, SHP2, and STK in promoting ovule identity.

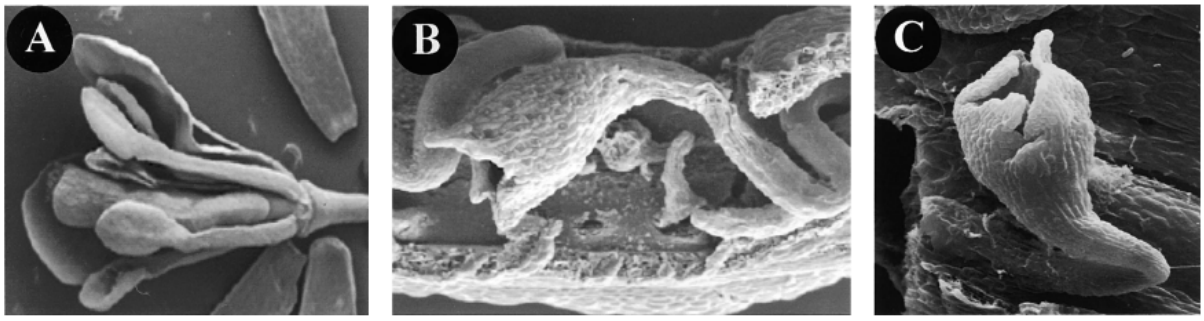


**Figure 5.** Phenotypes of the *SEP1/sep1 sep2 sep3* and *stk shp1 shp2* Mutant Plants.
**(A)** A flower of a *SEP1/sep1 sep2 sep3* plant. Flower development is normal in this mutant.

**Figure 5** (continued): **(B)** Ovules of a *SEP1/sep1 sep2 sep3* mutant plant. Ovule development is affected severely. Ovules are transformed into carpel- and leaf-like structures.**(C)** Ovules of a *stk shp1 shp2* plant. Ovule development is affected severely. The phenotype is similar to that shown in **(B)**.

AG and SEP probably form a stable complex with STK or one of the SHP proteins to promote ovule identity. Each of these complexes likely is enough to promote ovule identity. For instance, in the *shp1 shp2* double mutant, the complex composed of STK-SEP-AG probably is sufficient to promote normal ovule development. When the *STK*, *SHP1*, and *SHP2* genes all are inactive, the only possible complex that might be formed is an AG-SEP-AG complex, which presumably has reduced
ovule-promoting activity. The complexes composed of SHP and SEP proteins also likely are able to promote carpel identity in the absence of AG and AP2. In the *ag* single mutant, carpel formation is abolished completely. Because the *SHP* genes are induced by *AG*, these proteins are not present in this mutant to complement the absence of *AG*. However, in the *ag ap2* mutant, the activity of *SHP1*, *SHP2*, and *STK* is observed in the first-whorl organs, indicating that *ap2* directly or indirectly downregulates these genes. We could imagine that in the presence of these proteins in the first whorl of the *ag ap2* mutant, complex formation between SEP and SHP proteins promotes carpel identity and complexes of SEP, SHP, and STK proteins promote ovule identity.

**Ovule Development Is Affected in the *SEP1/sep1 sep2 sep3* Mutant**

SEP proteins have been shown to play key roles in the identity determination of petals and stamens by mediating the interaction between class-A and -B and class-B and -C, proteins, respectively (Honma and Goto 2001; Pelaz et al. 2001a). Furthermore, it has been proposed that they do not only function as mediators of protein interactions but also provide transactivation activity to the complex (Honma and Goto 2001). Our protein interaction data show that SEP proteins are able to establish interaction between proteins that promote carpel and ovule identity. Genetic evidence for a role of *SEP* genes during ovule development was not obtained, because the *sep* triple mutant completely lacks carpels. Interestingly, genetic titration experiments in which *SEP* gene activity was reduced showed that when *sep2* and *sep3* activity was abolished completely and only one *SEP1* allele was active, flowers developed rather normally but ovule development was affected severely, showing a clear role of *SEP* genes in ovule development. The observed phenotype was very similar to that of the *stk shp1 shp2* triple mutant, indicating that the genes with a major function in ovule development do not function without sufficient *SEP* activity. The protein complexes necessary for

proper ovule development, including SHP1, SHP2, and STK, probably are not formed sufficiently because of the low abundance of SEP protein. The same result was obtained when plants were generated in which only one *SEP2* allele was active, although one *SEP3* allele was enough to establish normal ovule development (data not shown), suggesting that *SEP3* is more efficient in promoting ovule identity. This difference in behaviour between the three *SEP* genes indicates that they probably are not completely redundant and therefore that these three duplicated genes might be preserved during evolution. This idea is strengthened further by the fact that *SEP3* expression is different from *SEP1/2* expression (Flanagan and Ma 1994; Savidge et al. 1995; Mandel and Yanofsky 1998).

**Evolutionary Conservation of STK–SEP Protein Interactions**

In several species, *STK* homologs have been identified: the *FBP7* and *FBP11* genes in petunia (Angenent et al. 1995), *OsMADS13* in rice (Lopez-Dee et al. 1999), and *ZMM1* and *ZAG2* in maize (Schmidt et al. 1993; Theissen et al. 1995). All of these *STK* homologs have more or less the same expression profile. Yeast two-hybrid protein interaction assays showed that the petunia proteins FBP7 and FBP11 interact with the SEP-like (AGL2-like) proteins FBP2, FBP5, and FBP9 (Immink et al. 2002). Interestingly, the rice MADS-box protein OsMADS13 also interacts with two SEP-like proteins, OsMADS24 and OsMADS45 (Immink et al. 2002). The evolutionary conservation of the MADS-box protein partners was confirmed by yeast two-hybrid exchange assays, which showed that the protein partners of FBP7 interact with OsMADS13 and vice versa (Favaro et al. 2002). We also performed yeast two-hybrid exchange assays between STK and the partners of OsMADS13, and these partners were exchangeable (data not shown). These results suggest that SEP proteins already had, early in evolution, before the division between monocot and dicot plants occurred, a role in MADS-box protein complex formation. Interestingly, SEP proteins also seem to play a role in facilitating nuclear localization, because in petunia, the FBP7 and FBP11 proteins were transferred to the nucleus only in the presence of the SEP-like proteins FBP2, FBP5, and FBP9 (Immink et al. 2002). It will be interesting to investigate whether the nuclear localization of STK also is dependent on the interaction with SEP proteins.

## METHODS

### Plant Materials

Arabidopsis thaliana ecotype Columbia was used for the *STK*, *SHP1*, and *SHP2* ectopic expression experiments. The plants were grown at 22˚C under long-day conditions (16 h of light/8 h of dark) in a mixture composed of 2.5:0.5 soil:vermiculite. The *ag-3* mutant ecotype Landsberg was provided by R. Sablowski (John Innes Centre, Norwich, UK).The genotype of the *ag-3*/_-derived plants was analyzed with a PCR introduced restriction site polymorphism (Jacobson and Moskovits 1991). DNA was amplified with the primers RSA-1 (5'-GTCGATTTCAGAAAATAAGAGCTC-3') and RSA-5 (5'-GAAGTATTACCCGAATCCGCCCCAAGAAG-3'), and the product was digested with BslI. Fragments amplified from the wild-type allele were digested, whereas the *ag-3* allele–derived PCR product was undigested. Fragments that differed by 20 bp were analyzed on a 3% agarose gel (2% low-melting-point agarose and 1% agarose).

### Binary Constructs and Arabidopsis Transformation

For ectopic *STK* expression in wild-type and heterozygous Arabidopsis *ag-3* mutant plants, the *STK* cDNA was cloned under the control of a double 35S promoter of *Cauliflower mosaic virus* (CaMV). The cDNA was amplified by PCR with the primers OL216 (5'-ATACCATGGGAAGAGGAAAGATAG-3') and OL217 (5'-CGGGATCCAGATTATCCGAGATGAAGAA-3') and cloned as a NcoI-BamHI fragment between the CaMV 35S promoter and a poly(A) terminator in a modified pUC19 vector. Introduced EcoRI and NcoI sites are underlined. The fragment containing the double CaMV 35S promoter, *STK* cDNA, and the poly(A) terminator was cloned as a AscI-PacI fragment in pCAMBIA1300. For ectopic *SHP1* and *SHP2* expression in Arabidopsis, the cDNAs were cloned under the control of a double CaMV 35S promoter. The *SHP1* open reading frame was amplified with OL525 (5'-AATTCCAGCTGACCACCATGGAGGAAGGTGGGAGTAGTCAC-3') and OL526 (5'-GATCCCCGGGAATTGCCATGTTACACAAGTTGAAGAGGAGGT-3'), and the open reading frame of *SHP2* was amplified with OL531 (5'-AATTCCAGCTGACCACCATGGAGGGTGGTGCGAGTAATGAA-3') and OL532 (5'-GATCCCCGGGAATTGCCATGTCAAACAAGTTGCAGAGGTGG-3'), and cloned in the Gateway overexpression vector pGD625 (derived from pGD120) (Immink et al. 2002) passing through pDONOR 201 (Life Technologies, Carlsbad, CA). Binary vectors were used to transform

*Agrobacterium tumefaciens* C58C1/pMP90 (Koncz et al. 1984). Arabidopsis plants were transformed using the floral dip method described by Clough and Bent (1998).

**Analysis of Arabidopsis Transformants**

The seeds derived from the T0 35S::*STK* transformants were selected on medium containing 20ug/ mL hygromycin, and after 2 weeks, seedlings were transferred to soil. Hygromycin-resistant plants were analyzed for the presence of the CaMV 35S::*STK* transgene by PCR using primers based on the CaMV 35S sequence (OL212; 5'-CTCGGATTCCATTGCCCAGCTAT-3') and on the *STK* sequence (OL218; 5'-TGGAGTTTTGAATCGTTTGGA-TGGAGTTTTGAATCGTTTGGA-3'). The seeds obtained from the T0 35S::*SHP1* and 35S::*SHP2* transformants were selected on medium containing 50_g/mL kanamycin, and after 2 weeks, resistant seedlings were transferred to soil. Kanamycin-resistant plants were analyzed for the presence of the transgene by PCR using the primers OL212 and OL526 for *SHP1* and the primers OL212 and OL532 for *SHP2*. *STK*, *SHP1*, *SHP*, *AG*, and *SEP3* expression was assayed by RT-PCR. RNA was extracted from leaves (Kater et al. 1998) and retrotranscribed with RT-Superscript II (Life Technologies). *STK*, *SHP1*, *SHP*, *AG*, and *SEP3* were amplified subsequently with specific primers and analyzed on agarose gels.

**Scanning Electron Microscopy**

Plant material was fixed overnight in 3% glutaraldehyde in 0.025 M phosphate buffer, pH 7, at 4_C, washed subsequently in 0.025 M phosphate buffer, pH 7, and incubated for 4 h in 1% osmic acid in 0.05 M phosphate buffer, pH 7. Samples were washed again in 0.05 M phosphate buffer, pH 7, dehydrated gradually in an ethanol series of 25, 50,70, 85, 95, and 100%, and dried in liquid carbon dioxide. Samples then were covered with gold, placed in a Nanotech sputter coater, and observed with a LEO 1430 scanning electron microscope (LEO Electron Microscopy, Thornwood, NY).

**Yeast Two- and Three-Hybrid Assays**

The two- and three-hybrid assays were performed in *Saccaromyces cerevisiae* strain PJ69-4A (James et al. 1996) as described previously (Davies et al. 1996). pBD, pAD, and pTFT1 (Egea-Cortines et al. 1999) vector constructs were selected on Yeast Synthetic Dropout (YSD) medium lacking Leu, Trp, and adenine, respectively. Three-hybrid interactions were assayed on selective YSD medium lacking Leu, Trp, adenine, and His supplemented with different concentrations of 3-aminotriazole (1, 3, 5, 10, and 20 mM). β-Galactosidase tests were performed according to (Davies

et al. 1996). Genes used for the yeast two- and three-hybrid assays were cloned in the Gateway vector GAL4 system (pDEST32 for binding domain fusions and pDEST22 for activation domain fusions) passing through pDONOR201 (Life Technologies). The cDNA of the genes was amplified by PCR with specific primers containing the attB1 and attB2 sequences for homologous recombination. Plasmids used as BD vectors were NOB249 for *STK*, NOB256 for *AG*, NOB250 for *SHP1*, and NOB284 for *SHP2*. Plasmids used as AD vectors were NOB246 for *STK*, NOB257 for *AG*, NOB247 for *SHP1*, NOB282 for *SHP2*, NOB283 for *SEP1*, and NOB245 for *SEP3*. pTFT1 was digested with EcoRI-SalI. *SEP3* cDNA was amplified with the primers OL340 (5'-CGGAATTCGGAAGAGGGAGAGTAGAATT-3')and OL304 (5'-CGCTCGAGTCAAATAGAGTTGTTGTCATAAGGTAACC-3'), digested with EcoRI and XhoI, and subcloned in pTFT1. Introduced EcoRI and NcoI sites are underlined.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for non-commercial research purposes. To obtain materials, please contact Lucia Colombo, lucia.colombo@unimi.it.

## ACKNOWLEDGMENTS

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# Chapter 4

# BASIC PENTACYSTEINE1, a GA Binding Protein That Induces Conformational Changes in the Regulatory Region of the Homeotic Arabidopsis Gene SEEDSTICK

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**ABSTRACT**

The mechanisms for the regulation of homeotic genes are poorly understood in most organisms, including plants. We identified BASIC PENTACYSTEINE1 (BPC1) as a regulator of the homeotic Arabidopsis thaliana gene SEEDSTICK (STK), which controls ovule identity, and characterized its mechanism of action. A combination of tethered particle motion analysis and electromobility shift assays revealed that BPC1 is able to induce conformational changes by cooperative binding to purine-rich elements present in the STK regulatory sequence. Analysis of STK expression in the bpc1 mutant showed that STK is upregulated. Our results give insight into the regulation of gene expression in plants and provide the basis for further studies to understand the mechanisms that control ovule identity in Arabidopsis.

**INTRODUCTION**

In multicellular organisms, organ identity is controlled by homeotic genes. In plants, most of these genes belong to the MADS-box gene family. In Arabidopsis thaliana, the MADS box gene family consists of 107 members, which can be divided over five subfamilies, namely MIKC, Mα, Mβ, Mγ, and Mδ (Parenicová et al. 2003). The plant MADS box genes that have shown to control organ identity at different stages of development all belong to the MIKC subfamily (Parenicová et al. 2003). Although it is known that floral organ identity and development are controlled through temporal and spatial activation and silencing of those types of transcription factors, little is known about their regulatory mechanisms. It has been shown that the expression of the MADS box genes AGAMOUS (AG), FLOWERING LOCUS C (FLC), and PLENA (PLE) are regulated by intragenic regions (Bradley et al. 1993; Sieburth and Meyerowitz 1997; Deyholos and Sieburth 2000; Sheldon et al. 2002). In particular, it has been shown that BELLRINGER (BLR) and LEAFY (LFY)bind to the second intron of AG. BLR prevents ectopic AG expression in the outer two whorls of a flower as well as in the reproductive shoot apical meristem (Bao et al. 2004), and LFY activates the expression of AG (Busch et al. 1999). Genetic and biochemical studies in organisms such as human, Saccharomyces cerevisiae, and Drosophila melanogaster have revealed that changes in expression patterns of several homeotic genes require remodeling of chromatin (Orphanides et al. 1999; Shimojima et al. 2003; Mellor and Morillon 2004). In Drosophila, homeotic genes like Ultrabithorax and Engrailed have shown to be regulated by GAGA binding proteins (GBPs), which

bind GA-rich elements (Biggin and Tjian 1989; Soeller et al. 1993) These GBPs interact with chromatin remodeling complexes like NURF and FACT and alter gene expression through these chromatin remodeling complexes (Lehmann 2004).In several plants species, genes have been identified that encode a class of proteins that also bind to GA-rich elements, like GBP in soybean (Glycine max) (Sangwan and O'Brian 2002),BARLEY B RECOMBINANT in barley (Hordeum vulgare) (Santi et al. 2003), and the BASIC PENTACYSTEIN (BPC) gene family in Arabidopsis, which includes seven members (BPC1 to BPC7; Meister et al. (2004)). The widespread expression patterns of those factors and the large number of potential target sequences present in plant genomes suggest that those proteins may affect expression of a variety of genes involved in different plant processes. Recently, MADS box genes that control ovule identity have been identified, which are SEPALLATA1 (SEP1), SEP2, SEP3, AG, SHATTERPROOF1 (SHP1), SHP2, and SEEDSTICK (STK) (Favaro et al. 2003; Pinyopich et al. 2003). Besides the control of ovule development, these genes have other functions, like floral organ development in general (SEP1/2/3) (Pelaz et al. 2000),carpel and stamen development (AG) (Bowman et al. 1989), fruit dehiscence (SHP1/2) (Liljegren et al. 2000), and development of the dehiscence zone at the funiculus (STK) (Pinyopich et al. 2003). Of these genes, only STK is expressed specifically in the septum and ovules (Rounsley et al. 1995; Pinyopich et al. 2003). Here, we report the analysis of the regulatory region of the homeotic MADS box gene STK. We show that its ovule- and septum-specific expression is controlled by regions comprising the first intron that is located in the 5’ untranslated region (UTR).Furthermore, we show that BPC1 binds these regions at multiple purine-rich sites, and using tethered particle motion (TPM)technology (Finzi and Dunlap 2003), we demonstrate that these multiple sites are used to induce conformational changes in the STK regulatory regions. By analyzing the expression of STK in a bpc1 mutant, we revealed that STK expression is upregulated in this mutant.

## RESULTS

### Analysis of the STK Regulatory Region

To identify the regions that contain the regulatory elements that control the ovule- and septum-specific expression of STK, a 2.8-kb sequence upstream of the ATG was cloned and fused to the b-glucoronidase (GUS) reporter gene. This fragment contains a region of 1.4 kb 59 of the transcription start site and a region of 1.4 kb containing the 5’ UTR and the first intron of 1.3 kb.

Analysis of 20 transgenic plants transformed with this construct showed that the 2.8-kb fragment contains all the information to give specific expression in ovules and septum (Figure 1A). To test whether the 1.3-kb intron contains regulatory elements necessary for specific STK expression, we transformed Arabidopsis plants with a construct in which the intron was deleted. These transgenic plants showed GUS expression in all floral organs(Figure 1B), demonstrating the importance of the first intron for ovule- and septum-specific STK expression.

**BPC1 Interacts with the Regulatory Regions of STK**

To identify factors that interact with the promoter region of STK, a yeast one-hybrid screening was performed. We divided the STK regulatory region in six parts of ~450 bp and fused these to the His and LacZ reporter genes. These constructs were integrated in the

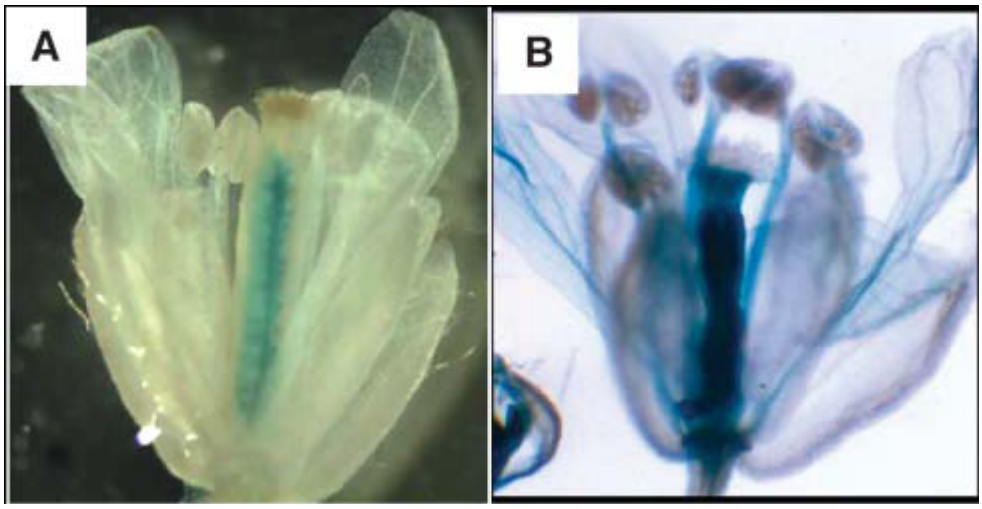


**Figure 1.** GUS Expression in Arabidopsis Flowers.

**(A)** Flower from a plant containing the promoter of STK fused to the GUS coding sequence, showing ovule- and septum-specific GUS expression.

**(B)** Aspecific GUS expression throughout the entire flower from a plant containing only the 5'-flanking region without intron region in the 5'UTR of STK fused to the GUS coding sequence.

genomes of the yeast strains Y187 (His construct) and YM4271 (LacZ construct). The Y187 strain was mated with yeast strain AH109 containing a whole plant cDNA library. The plasmids extracted from the colonies that were able to grow on medium lacking His were transformed to the YM4271 strain to test the activation of the LacZ reporter gene. This experiment resulted in the identification of BPC1 (Sangwan and O'Brian 2002), which interacted with two fragments that comprise sequences 5' of the transcription start site, a part of the 5' UTR and a part of the leader intron

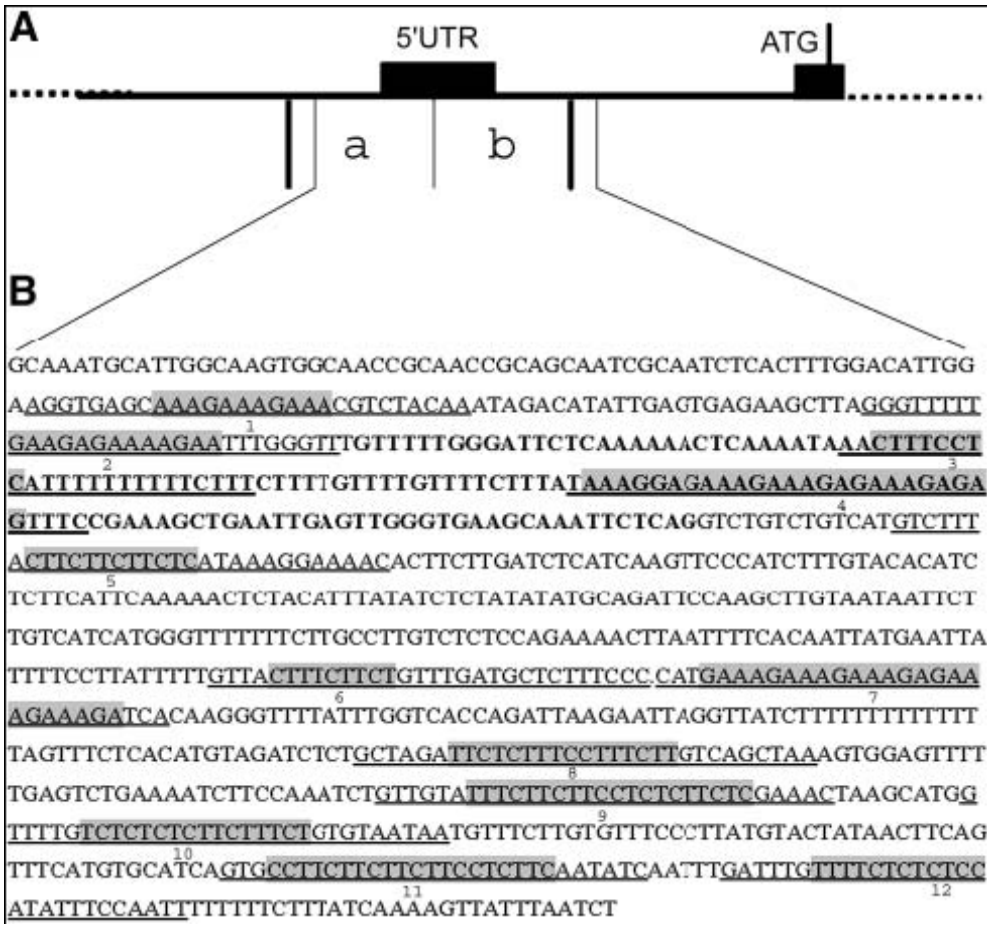


**Figure 2**. Schematic Representation of the STK Promoter.

**(A)** Promoter/intron region with the two regions (a and b) that were binding BPC1 in the yeast one-hybrid screen. ATG, start codon.

**(B)** Sequence of the region of the STK promoter that contains 12 GA-rich regions (shaded, numbered 1 to 12). The underlined sequences are the oligonucleotide sequences used in the EMSA experiments. The location of the exon is displayed in bold.

(Figure 2A, fragments a and b). The identified BPC1 cDNA clones encoded three BPC1 variants with different truncations at the N-terminal part of the protein, resulting in proteins of 145, 120, and 111 amino acids, respectively. These results indicate that the C-terminal part of the protein is sufficient for DNA binding, similar to what was previously reported for BPC2 (Meister et al. 2004). BPC1 and similar proteins of Arabidopsis, soybean, and barley have shown to bind DNA at sites that contain GA repeats (Sangwan and O'Brian 2002; Santi et al. 2003; Meister et al. 2004). Analysis of the STK promoter sequences, to which BPC1 binds, revealed 12 regions containing at least eight nucleotides with only purines (shaded regions in Figure 2B). To test whether full-length BPC1 is able to bind to these putative binding elements, electromobility shift assays (EMSA's)

were performed. We designed double-stranded oligo nucleotides of ;30 bp, containing the putative binding sites (Figure 2B), and labeled element 4 radioactively. As shown in Figure 3A, when we added 10 ng of BPC1 protein, two shifted bands were produced. To test the specificity for element 4, unlabeled double stranded element 4 was added to compete with the labeled probe. As can be seen in Figure 3A, a 100-fold molar excess was sufficient to out-compete the labeled oligo nucleotide almost completely. The affinity for the other putative elements was tested by competition EMSA assays using labeled element 4. Elements 1, 7, 9, 8, 10, and 12 were able to compete with element 4 (increasing affinity),whereas elements 2, 3, 5, 6, and 11 were not (Figure 3B; data not shown).Until now, BPC proteins have shown to bind GA repeats of 12 nucleotides long (GA)6 (Meister et al. 2004). Our experiments suggest that BPC1 also binds to sequence motifs other than a repetition of multiple GA repeats because BPC1 is able to bind elements 4, 7, and 8, which contain only a double GA repeat (Figure 3D). Furthermore, element 1 can interact with BPC1 even if it does not contain any direct GA repeat. From an alignment of BPC1 binding sites in the STK promoter, a consensus binding sequence could be predicted, consisting of nine purines: RGARAGRRA (Figure 3D). The affinity of BPC1 for the consensus was greatly reduced when one of the purines was replaced by a pyrimidine, like shown for nucleotide 7 in Figure 3C. In the case of nucleotide 6 of the consensus, even a change of G to A is sufficient to abolish the affinity because BPC1 was not able to bind to element 2 (Figure 3E).

**BPC1 Induces Conformational Changes in the STK Regulatory Region**

The presence of multiple binding sites for BPC1 in the STK regulatory region indicates that this protein might form a complex inducing architectural changes in the DNA. To test this, we used a single-molecule optical microscopy technique called TPM analysis (Finzi and Dunlap 2003).

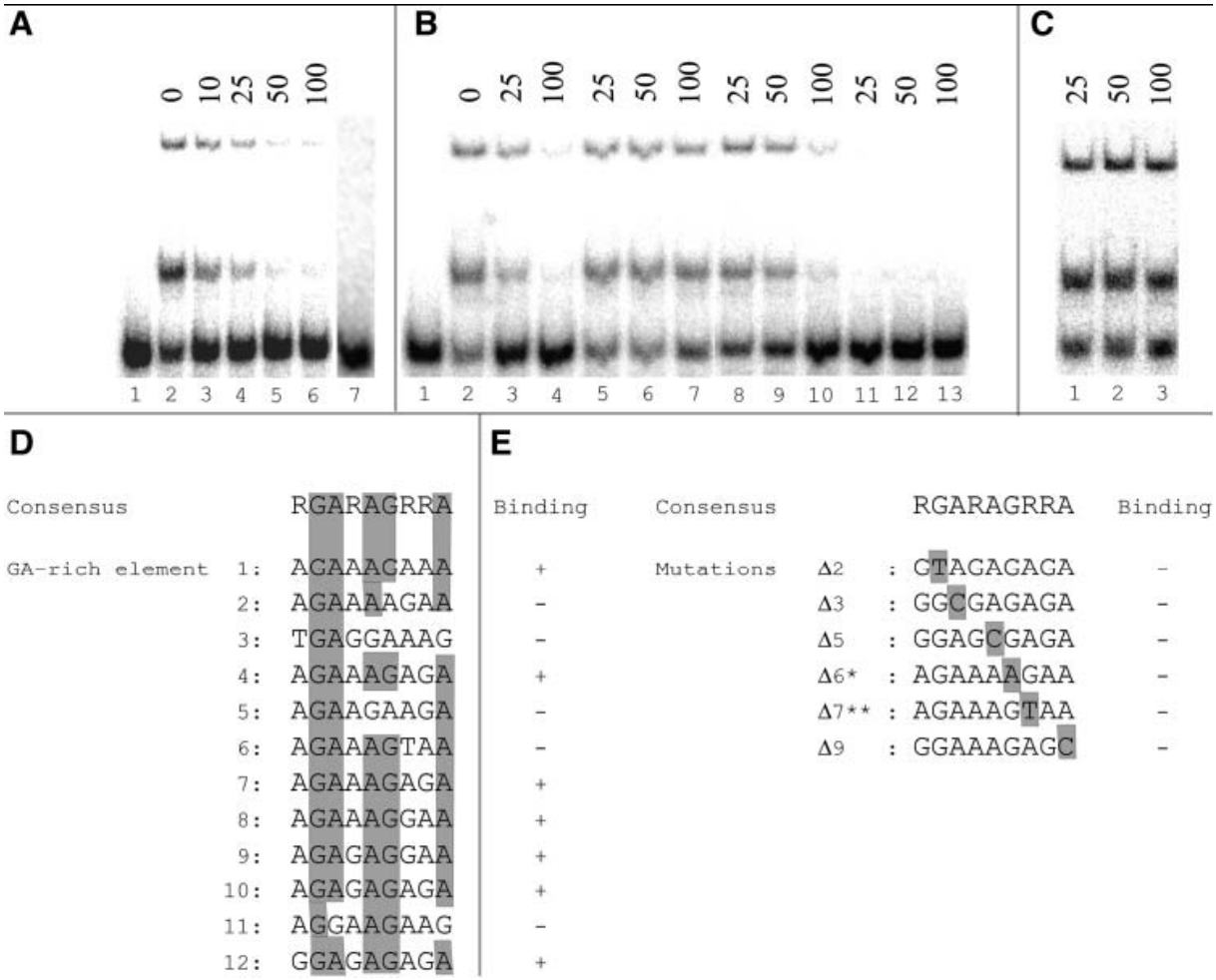


**Figure 3**. Analysis of BPC1 Binding to GA Elements. EMSA competition assays using labeled element 4 as a probe. The molar excess of the competitor is indicated above the figures. **(A)** Unlabeled element 4 is used as a competitor (lane 1 is free probe). A 100-fold excess of unlabeled element 4 out competes the labeled probe completely. Lane 7 is a control using the unrelated maltose binding protein, no band shift is observed. **(B)** Competition assays using unlabeled element 4 (lanes 2 to 4), element 2 (lanes 5 to 7), element 9 (lanes 8 to 10), and element 12 (lanes 11 to 13) as a competitor, showing that elements 4, 12, and 9 are bound by BPC1, whereas element 2 is not. **(C)** Competition assay using mutated element 12 (nucleotide 7, A to T) as a competitor. The affinity of BPC1 for the mutated element is completely lost because of this mutation **(D)** Alignment of the different elements that were able to bind BPC1 and were not able to bind BPC1, leading to the shown consensus.**(E)** Point mutations compared with the consensus, which lead to the decrease in binding capacity of BPC1. *, sequence as present in element 2 in Figure 3D; **, sequence as present in element 6 in Figure 3D.

TPM allows direct observation of protein-induced significant DNA conformational changes, such as bending or looping. This is possible by observing the Brownian motion of a bead tethered to a microscope flow-chamber by a single DNA molecule. The range of the Brownian motion will change as a consequence of any alteration in the DNA end-to-end distance, such as those observed

as a consequence of protein-induced architectural changes. We analyzed a STK promoter fragment of 1413 bp in which all the seven boxes that bind BPC1 are present. TPM analysis showed dynamic transitions between different DNA conformational states (Figure 4A). The experimental traces present step-like changes in the end-to-end distance of the DNA molecules. This indicates that BPC1 can induce loops of variable size in the DNA, which are in a dynamic equilibrium. A particularly stable one shortens the DNA end-to-end distance by; 50 to 60 nm. Because BPC1-mediated interaction between boxes 4 and 12 would decrease the DNA end-to-end distance by the same amount and these boxes are those that show strong binding of BPC1, as revealed by EMSA assays, we suggest that this is the dominant interaction. To determine whether box 4 and box 12 are enough to establish the observed conformational change, we constructed a fragment in which only these two boxes are present at the same distance. The two fragments were analyzed simultaneously in the same TPM measurement by visually distinguished labeling of both fragments with beads of different sizes. This experiment showed that upon addition of BPC1, the fragment that contains only boxes 4 and 12 does not show any decrease in DNA end-to-end distance (Figure 4B), whereas for the control fragment, a shortening of ;50 to 60 nm was observed. This result clearly indicates that cooperative binding to multiple BPC1 binding sites is necessary for the induction of conformational changes in the STK regulatory region. To show that the observed end-to-end reduction is specific to BPC1 and not caused by the presence of an unrelated protein, we also performed TPM assays with the maltose binding protein (MBP). As shown in Figure 4C, no end-to-end distance reduction was observed.

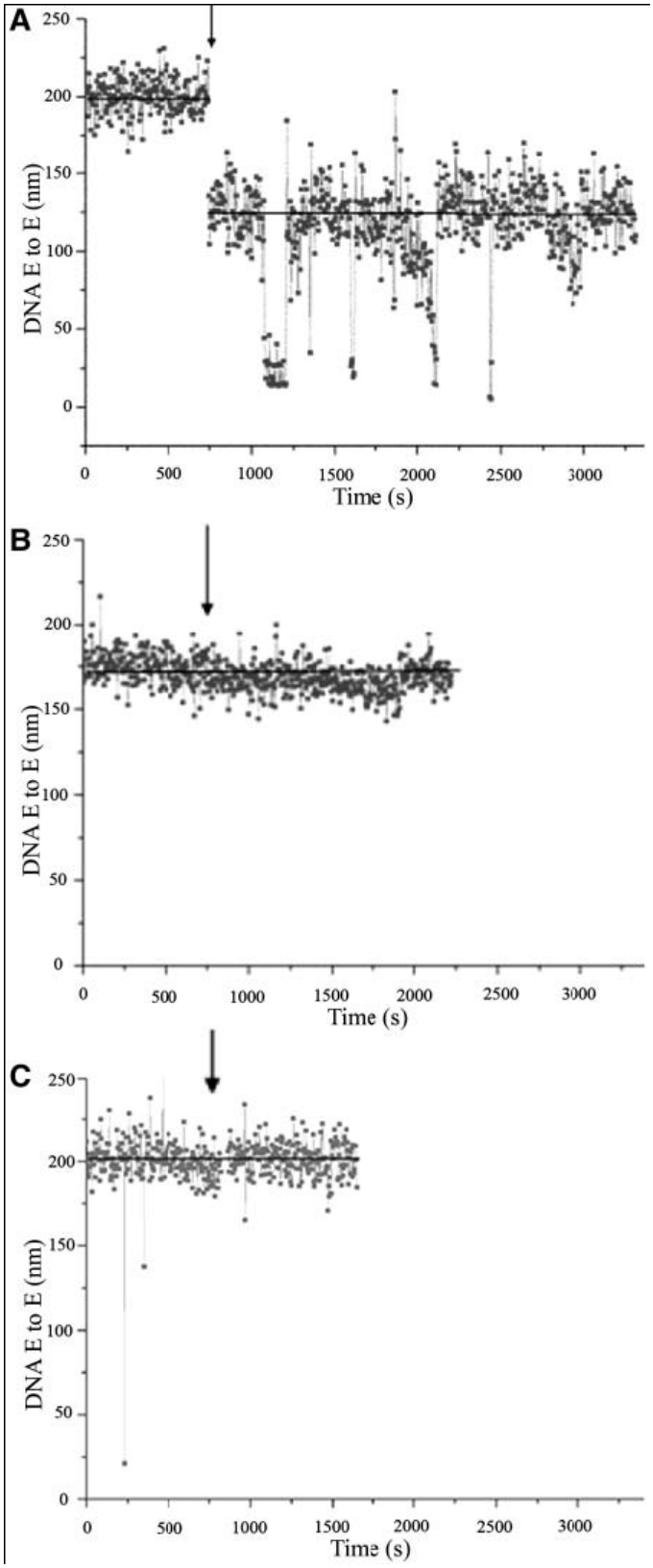


**Figure 4.** TPM Analysis of BPC1 Interaction with the STK Promoter. DNA end-to-end distance (E to E) measured in TPM experiments. The trace shows the variations in time of the DNA end-to-end distance before and after addition of

**Figure 4.**(continued) the protein. The arrow indicates the time of addition of protein. The black line shows the average value of the DNA end-to-end distance before and after addition of protein. **(A)** The DNA fragment used is 1413 bp long and contains BPC1 binding sites 1 through 12; the distance between box 12 and the digoxigenin label is 240 bp, whereas the distance between site 1 and the biotin label is 320 bp. The end-to-end distance after addition of BPC1 is reduced by; 50 to 60 nm. **(B)** DNA fragment used contains only boxes 4 and 12; the distance between boxes 4 and 12 is approximately like the distance in the otherDNA fragment. The same holds for the distance between these boxes and the digoxigenin and biotin labels. The addition of BPC1 does not change the DNA end-to-end distance of the DNA. **(C)** Control experiment using the unrelated MBP. The DNA fragment isthe same as used in the experiment represented in Figure 4A. No alteration of the DNA end-to-end distance after the addition of the MBP was observed.

## BPC1 in the Control of STK Expression

It has been shown by RT-PCR analysis that BPC1 is expressed throughout the Arabidopsis plant (Meister et al. 2004). To investigate the BPC1 expression in more detail during Arabidopsis flower development, we performed in situ hybridizations. This analysis revealed that BPC1 expression is visible in the floral meristem and floral organ primordia (Figure 5A). At later stages, BPC1 remains expressed in all floral organs and in particular in the ovule (Figures 5B and 5C).To investigate whether BPC1 regulates STK expression, we searched the Salk T-DNA collection (Alonso et al. 2003) for BPC1 insertion mutants. In this collection, we identified a line that has a T-DNA insertion in the first intron 293 bp upstream of the ATG. Homozygous mutant lines were identified by PCR and DNA gel blot analysis. Subsequent RNA gel blot analysis (data not shown)and in situ hybridization (Figure 5D) confirmed the absence of BPC1 expression in this mutant. Phenotypic analysis of homozygous bpc1 mutant plants during different stages of development revealed no alterations with respect to wild-type plants. To analyze whether the absence of BPC1 affects STK expression, we performed real-time RT-PCR on RNA extracted from Arabidopsis flowers of wild-type and bpc1 mutant plants. This analysis showed that the STK expression in the bpc1 mutant is increased by approximately threefold with respect to the wild-type (Figure 6).To understand whether there are also changes in the tissue specificity of STK expression, we performed in situ analysis on flowers of wild-type and bpc1 mutant plants using a STK-specific probe. This analysis showed that STK expression remained ovule and septum specific (Figures 5E and 5F) like what was shown for the STK expression in wild-type flowers (Figure 1A; Pinyopich et al. (2003)).

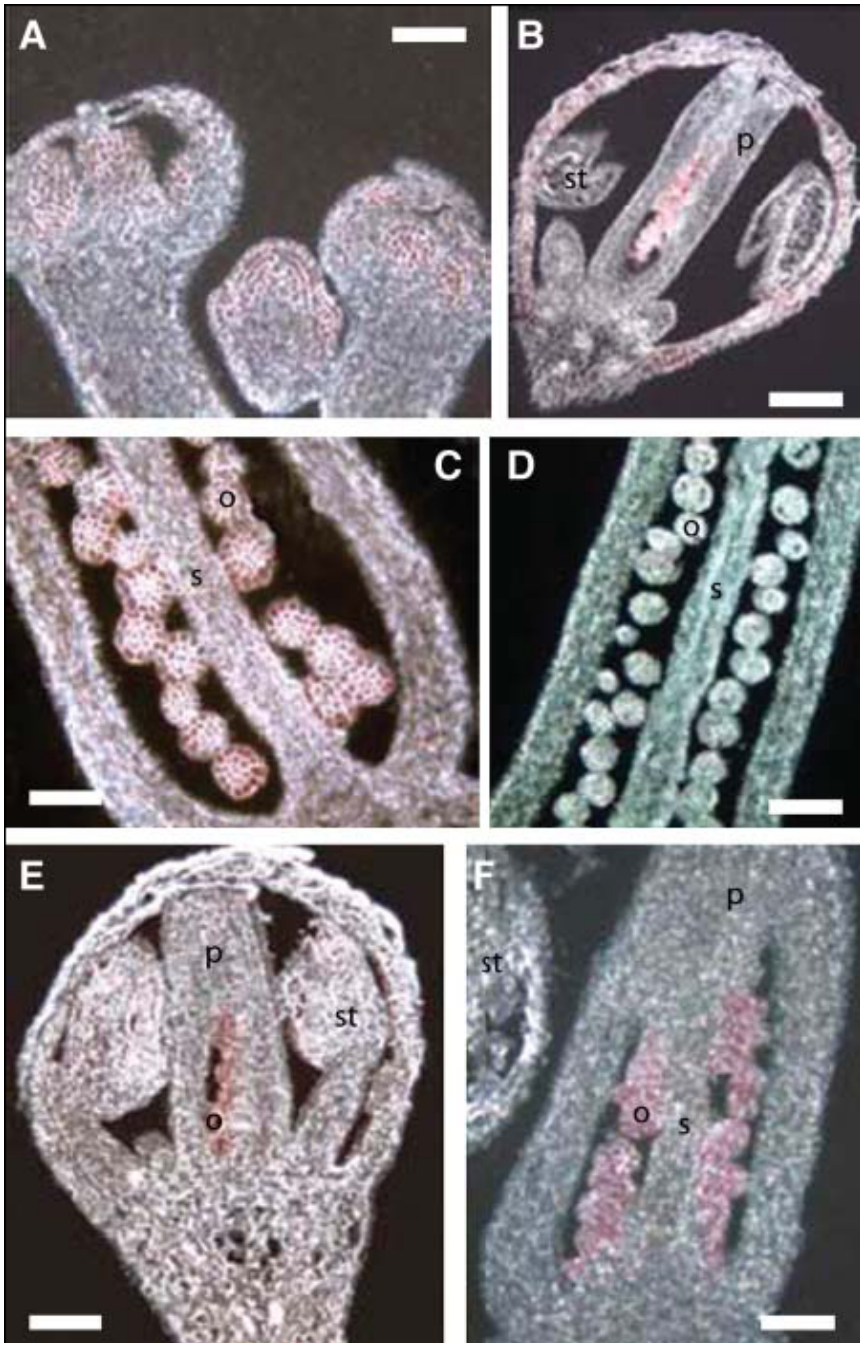


**Figure 5.** STK and BPC1 Expression Analysis in Wild-Type and bpc1 Mutant Arabidopsis Flowers by in Situ Hybridization.**(A)** In situ hybridization on very young flowers showing BPC1 expression in the floral meristem and floral organ primordia.**(B)** Expression of BPC1 in a flower showing expression in all floral organs and especially in ovules.**(C)** Expression of BPC1 in ovules.**(D)** In situ hybridization on a bpc1 mutant flower using the BPC1 probe, showing no expression.**(E)** and **(F)** In situ hybridization on bpc1 mutant flowers showing STK specific expression like in wild-type flowers. s, septum; st, stamen; p, pistil; o, ovule. Bars in **(A)** and **(C)** = 20 mm; barsin **(B)** and **(D)** to **(F)** = 40 mm.

## DISCUSSION

Homeotic genes control the identity of organs. In plants, the best studied homeotic genes control floral organ identity and belong to the MADS box gene family. Misexpression or defects in the timing of the expression of these genes causes homeotic transformations of floral organs and severe aberrations in flower development. Here, we report on the regulation of the ovule identity gene STK, a MADS box gene that is specifically expressed in ovules and septum.

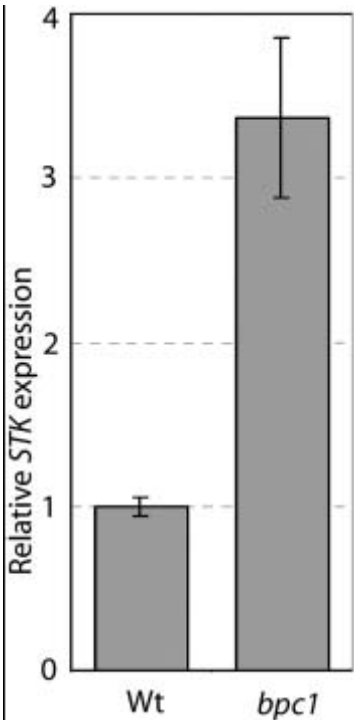


**Figure 6.** STK Expression Analysis in Wild-Type and bpc1 Mutant Arabidopsis Flowers by real-time RT-PCR. Error bars represent standard deviations calculated on five different replicas. STK is approximately three times upregulated in the bpc1 mutant with respect to the wild type.

### The STK First Intron Contains Elements for Ovule-Specific Expression

To identify the region that regulates STK expression, we cloned a 2.8-kb fragment upstream of the ATG. This fragment contains the 5'UTR with a large intron and a 5'-region flanking the transcription start site. Using the GUS reporter gene, we showed that this fragment is enough to drive specific GUS expression in ovules and septum. Subsequent studies revealed that deletion of the first intron resulted in the loss of ovule-specific expression. Other MADS box genes, such as PLE, AG, and FLC, have also shown to be regulated by intron sequences (Bradley et al. 1993; Sieburth and Meyerowitz 1997; Deyholos and Sieburth 2000; Sheldon et al. 2002). The AG second intron has been shown to be sufficient to regulate AG-specific expression (Deyholos and Sieburth

2000). Analysis of the AG intron combined with genetic experiments revealed that binding sites for AP2, LEUNIG, LFY, WUSCHEL, and MADS-box proteins are present (Drews et al. 1991; Busch et al. 1999; Hong et al. 2003). Analysis of the first STK intron sequence does not reveal a priori any transcription factor binding site.

**BPC1 Binds to the STK Regulatory Region**

Using the yeast one-hybrid system we identified BPC1, which binds the promoter region of STK. BPC factors have only been identified in plants and their name is due to the presence of an unusual arrangement of five Cys residues in the highly conserved C terminus. The Arabidopsis genome sequence contains seven BPC genes that can be subdivided into three classes, of which BPC1 groups together with BPC2 and BPC3 based on overall sequence homology (Meister et al. 2004). Also, in barley, soybean, rice (Oryza sativa), potato (Solanum tuberosum), and tomato (Lycopersicon esculentum), BPC-like proteins that bind sequences rich in GA-repeats have been identified based on experimental data (soybean and barley) and homology data (rice, potato, and tomato) (Sangwan and O'Brian 2002; Santi et al. 2003; Meister et al. 2004). The experimental data published so far showed that these proteins in Arabidopsis, soybean, and barley bind to $(GA)_6$, $(GA)_8$, and $(GA)_9$ repeats, respectively (Sangwan and O'Brian 2002; Santi et al. 2003; Meister et al. 2004). In this study, we show that BPC1 is also able to bind to non direct GA repeats, like GA-rich element 1. By aligning the sites in the STK promoter that bind and do not bind BPC1 and performing mutagenesis experiments, we could define a 9-bp DNA consensus sequence (RGARAGRRA) for BPC1 binding. For BPC2 of Arabidopsis and the homolog from barley, it has been shown that the highly conserved C-terminal part of the protein, which contains a putative zinc-finger motif, is necessary for DNA binding (Santi et al. 2003; Meister et al. 2004). Our one-hybrid experiments confirmed these results because an N-terminal deletion reducing the protein to 111 amino acids was still able to bind the STK promoter. These 111 amino acids contain the conserved domain, including the putative zinc-finger. Further experiments will have to show if these proteins really function as zinc-finger proteins. An indication that BPC proteins contain a zinc-finger motif that might be involved in DNA binding comes from the observation that the GBPs of Drosophila (based on primary amino acid sequence, structurally unrelated to the plant BPC proteins) do contain a zinc-finger motif that has been shown by NMR studies to contact the DNA at GAGA repeats (Omichinski et al. 1997). To investigate whether BPC1 is able to induce conformational changes in the STK regulatory region, we performed TPM analyses. This method allows the study of

conformational changes at the level of single DNA molecules by measuring the Brownian motion of a micro-sphere. In our study, we used two microspheres of different size, which allowed us to compare directly the behavior of two different DNA molecules before and after addition of BPC1 protein. With this method, we showed that BPC1 is able to induce conformational changes when all the GA-rich elements in the STK regulatory sequence are present. However, when only the two strong binding sites are available that seem to be involved in a major conformational change in the wild-type DNA construct, no conformational changes are observed (Figure 4B). We took this as evidence that cooperative binding to multiple BPC1 binding sites may be necessary to have a significant DNA reconfiguration by BPC1. This TPM analysis indicates that BPC1 induces conformational changes in the STK promoter by forming a multimeric complex that binds to multiple sites. This idea is strengthened by the observation that in the EMSA assays, two shifted bands are always observed that could be attributable to the formation of a higher-order complex. The rice STK homolog OsMADS13 has an expression profile similar to that of STK (Lopez-Dee et al. 1999). Analysis of sequences upstream the ATG (which also includes an intron) showed that this region contains only three GA-rich elements that correspond to the consensus binding site for BPC1. This might implicate that the regulation of these genes has not been conserved between these species or that the affinity of rice BPC1-like proteins for this consensus has slightly changed because six additional GA-rich elements are present in the regulatory region of OsMADS13 that, as a result of one mismatch, do not have affinity for BPC1.

**BPC1 in the Control of STK Expression**

BPC1 induces conformational changes in the STK promoter region. To understand which role BPC1 plays in STK regulation, we analysed STK gene expression in the bpc1 T-DNA insertion mutant by real-time PCR. This analysis showed that STK is approximately three times upregulated in the bpc1 mutant, indicating that BPC1 functions as a repressor of STK. However, considering the expression data showing that BPC1 is expressed in all parts of the plant and especially in ovules, it seems not very obvious that BPC1 functions as an active repressor of STK. A more credible hypothesis might be that BPC1 induces conformational changes that allow the recruitment of a specific regulatory complex. BPC1 is probably redundant with BPC2 (and maybe also BPC3) in regulating STK and other genes (Meister et al. 2004). However, BPC1 and BPC2 are not completely identical, and the conformation of the STK promoter might be different when only BPC2 is recruited to the purine-rich boxes. They also might recruit different types of factors to the

STK promoter. Therefore, it could well be that the absence of BPC1 causes this relative mild effect on STK expression as a result of architectural and/or composition changes in transcription factor complexes. This hypothesis also fits more with the observations reported by Meister et al. (2004). They showed that BPC proteins bind to the promoter of INNER NO OUTER (INO). INO encodes a member of the YABBY family of transcription factors that regulates abaxial–adaxial patterning in Arabidopsis ovules (Villanueva et al. 1999). The regulatory region of INO contains six GA-rich elements. Deletion of BPC binding elements in the INO promoter resulted in a reduction or complete loss of expression, indicating that BPC proteins are likely not working as repressors. From our analysis, it is also clear that the absence of BPC1 does not result in a change in expression profile because our in-situ data showed that specificity of STK expression is retained in the bpc1 mutant. This is likely due to redundancy with BPC2 and maybe also BPC3. Future studies on double or triple mutants have to clarify this further.

## METHODS

### GUS Constructs and Assay

Promoter fragments were cloned into pBluescript SK+ containing the GUS coding sequence and a Cauliflower mosaic virus–poly(A). The promoter fragments were amplified using primers OL152 (5'-GCTCTAGATGTTGGGTATGTTCTCACTTTCTTG-3') and OL166 (5'-TCTTCTCATGATTCCATTTTAAACATCAAAC-3'). The region 5' of the transcriptionstart was amplified using primers OL152 and OL167 (5'-TCTTCATGATTCAGCTTTCGGAAACTCTC-3'). The intron region was amplifiedusing primers OL467 (5'-GCTCTAGATGAAGCAAATTCTCAGGTCTGTC-3') and OL468 (5'-CGGGATCCTCTTCCGATCCTCATTTTAAACATC-3'). The promoter-GUS and the promoter without intron region-GUS cassettes were cloned into pCAMBIA1300-H. Arabidopsis thaliana(ecotype Columbia) was transformed with these constructs using the Agrobacterium tumefaciens–mediated floral dip method (Koncz et al. 1984; Clough and Bent 1998). The GUS assays were performed as described (Liljegren et al. 2000).

### Yeast One-Hybrid Experiments

The STK regulatory region was divided in six overlapping fragments of ;400 bp. The fragments to which BPC1 binds were amplified using primers OL734 (5'-

GCGAATTCTACTAATACTTTATATGTGCGATTTAGC-3') and OL735 (5'-GCGAGCTCGGTACCAATTCAGCTTTCGGAAACTCTC-3') for fragment A and primers OL876 (5'-GGAATTCTCAGGTCTGTCTGTCATGTC-3') and OL877 (5'-GGCTCGAGTCTAGAGAGGAAGAAGAAATACAACAG-3') for fragment B. The amplified fragments were cloned into the pLacZi and pHISi vectors (Clontech, Palo Alto, CA),which were subsequently cut with ApaI (pLacZi) or XhoI (pHISi) and used to transform yeast strain YM4271 (pLacZi) or Y187 (pHISi). The whole plant cDNA library, which was cloned in pGADT7-rec and introduced into yeast strain AH109, was mated with Y187 as described in the Clontech user manual and selected on medium lacking His with 35 mM 3-amino1,2,4-triazol. Plasmids were extracted from positive colonies and retransformed to YM4271 for b-galactosidase (LacZ) assays (Duttweiler 1996).

**EMSA**

The BPC1 protein was produced by cloning the coding sequence, whichwas amplified using primers OL828 (5'-GAGGATCCATGGACGATGATGGATTTCGC-3') and OL829 (5'-GAGTCGACTTATCTGATCGTGACAAACTTATTGG-3'), into the pMAL-c2X vector (New England Biolabs,Beverly, MA). Oligo-nucleotides were end-labeled with polynucleotide kinase (Roche, Monza, Italy). Ten nanograms of the BPC1-MBP fusion protein were used per experiment and incubated for 20 min with the labeled double-stranded oligo-nucleotides at room temperature in binding buffer (0.5 mM EDTA, 50 mM NaCl, 5 mM MgCl2, 20 mM Tris-HCl, pH 8.8, 1 mM DTT, 5% glycerol, 0.5 mg/mL BSA, and 5 ng poly dIdC). The samples were run on 8% polyacrylamide gels (37.5:1).

**TPM**

The TPM experiments were performed as described by Finzi and Dunlap (2003). The DNA tethers were constructed via PCR using a plasmid containing the STK promoter. The primers that were used are as follows: bio-5'-TTGGTCTTGCCGTGAACTTGG-3' and dig-5'-AATTTTGACCCATCCCGTGTAC-3', where ''bio'' means biotin and ''dig'' means digoxigenin. The DNA fragment was attached to the micro-chamber by anti-digoxigenin antibodies that were coated to the glass of a micro-chamber. The beads that were coated with streptavidin were subsequently attached to the biotin label at the other end of the DNA (for details, see Finzi and Dunlap (2003)). Different DNA fragments in the same micro-chamber were labeled with beads 0.2

and 0.4 mm in diameter, respectively. BPC1 was used at a concentration of 0.037 ng/mL. The buffer in which the experiment was performed contains 10 mM Tris-HCl, 200 mM KCL, 5% DMSO, 0.1 mM EDTA, 0.2 mM DTT, and 0.1 mg/mL a-casein.

**DNA Gel Blot Analysis**

The bpc1 mutant was obtained from the SALK lines (SALK_072966.43.30.x) (Alonso et al. 2003) and ordered from the Nottingham Arabidopsis Stock Centre (Scholl et al. 2000). The T-DNA insertion was confirmed by DNA gel blot analysis, using 1.5 mg of DNA, which was cutwith XbaI. The DNA was loaded on a 0.8% agarose gel and run overnight at 30 V. DNA transfer to Hybond-N+ and hybridizations were performed according to the manual (Amersham Biosciences, Cologno Monzese, Italy). The probe was obtained by PCR on genomic DNA using primers AtP240 (59-GCTTCGATGAGAAGATCGCTAG-39) and AtP241 (59-GAATATGAGTCCACTGGACG-39) and subsequent purification from gel using the Nucleospin extract kit (Machery-Nagel, Dűren, Germany). The probe was labeled using the random-primed DNA labeling kit from Roche.

**Expression Analysis**

Total RNA was extracted from Arabidopsis flowers (Verwoerd et al. 1989) and subsequently treated with DNaseI. RNA from wild-type and bpc1 mutant plants (obtained from a segregating population) was reverse transcribed using the Bio-Rad i-Script cDNA synthesis kit (Milan, Italy). For quantitative real-time PCR, the iCycler iQ real-time PCR detection system (Bio-Rad) was used. cDNA amplifications were performed using the iQ SYBR-Green Supermix (Bio-Rad). As a control for the presence of genomic DNA contamination, we performed a real-time PCR on RNA after DNase treatment. The primers used for STK are 5'-GTTCTGATAGCACCAACACTAGC-3' and 5'-ACTCATGCTTCTTGGACCTGATC-3'. The data were normalized to actin, amplified with primers 5'-CCAATCGTGAGAAAATGACTCAG-3' and 5'-CCAAACGCAGAATAGCATGTGG-3'. All PCR reactions were performed twice in triplicates. The standard curves were constructed using serial cDNA dilutions. The PCR efficiency was close to 100%; relative gene expression was determined using the 2-ΔΔCt method (Livak and Schmittgen 2001). For the in situ hybridization, Arabidopsis flowers were fixed and embedded in paraffin as described previously (Lopez-Dee et al. 1999). Digoxigenin-labeled gene-specific antisense RNA

probes were generated by in vitro transcription following the instructions of the in vitro transcription kit (Roche). Hybridization and immunological detection were performed as described previously (Lopez-Dee et al. 1999).

**ACKNOWLEDGMENTS**

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# Chapter 5

# Characterisation of the BASIC PENTACYSTEINE type I genes

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**Introduction**

In Drosophila homeotic or *Hox* genes play an important role in the determination of the organ identity. The expression of these genes are tightly regulated by the stable modifications of chromatin structures. Two groups of regulators have been identified, namely *Polycomb Group* (*PcG*) and *Thritorax Group* (*TrxG*). The first group is involved in the silencing of Hox genes and the *TrxG* is required for the maintenance of the *Hox*-gene expression (Lehmann 2004). One of the *TrxG* genes *Tritorax-like* (*Trl*), encodes GAGA-factor (GAF), a protein that is able to bind GAGA-elements. This protein does not only co-immunoprecipitate with Tritorax-response elements (TRE's) and the Tritorax protein complex (Poux et al. 2002), but also with Polycomb Response Elements (PRE's) (Horard et al. 2000), suggesting that GAF is involved in the regulation of *Hox*-gene expression, through co-binding with activators and repressors.
The regulation occurs through nucleosome remodelling, by binding of GAF to Nucleosome Remodelling Factors (NURFS) (Xiao et al. 2001) and Facilitates chromatin transcription (FACT) (Shimojima et al. 2003). GAF also interacts with SAP18, a protein present in the Histone deacetylase complex (HDAC)(Zhang et al. 1997). These interactions lead to the activation or repression of target genes, which are frequently found to be homeotic genes.

In Arabidopsis little is known about the regulation of homeotic genes. However a tight regulation of these genes is very important, since the absence or ectopic expression of these genes often result in homeotic conversions. One of the best studied homeotic genes is *AGAMOUS (AG)* (Yanofsky et al. 1990). Regulation of this gene is mediated mainly by it's second intron, where binding sites for several transcription factors are present. *BELLRINGER* (*BLR*) and *APETALA2* are known to repress *AG* in the outer two whorls of Arabidopsis flowers (Bomblies et al. 2003; Bao et al. 2004), and recent publications showed that also complexes formed between LEUNIG (LUG), SEUSS (SEU), APETALA1 (AP1) and SEP3 (Sridhar et al. 2004), as well as complexes formed between LUG, SEU, AGL24, SVP and AP1 repress the expression of AG (Gregis et al. 2006). In the inner two whorls the expression of *AG* however is activated by proteins like AG, SEP3, LFY and WUS (Castillejo et al. 2005).
*AG* contains several binding sites for the BASIC PENTACYSTEIN protein family, which is a small family of transcription factors that are able to bind to purine rich sequences. This family consists of 7 members which have been divided in 3 subfamilies, type I (*BPC1-3*), type II (*BPC 4-6*) and type

III (*BPC7*) (Meister et al. 2004). Homologous genes also exist in other plant species like Barley (BBR), soy bean (GmGBP), rice (AY569037) (NCBI), and tomato (TC85862) (TIGR) (Santi et al. 2003). All the identified BPC's contain a highly conserved basic C-terminus containing 5 highly conserved cysteins. This C-terminus contains a putative Zinc finger, which is, together with the binding of GA-rich sequences, the only common feature between these proteins and the GBP's from Drosophila and human.

In Arabidopsis BPC1 has been shown to regulate the expression of the homeotic gene *SEEDSTICK (STK)*. In the *STK* regulatory region there are several purine-rich sequences present which are able to be bound by the BPC1 protein (Kooiker et al. 2005).

It has been shown by TPM that by cooperative binding BPC1 is able to change the conformation of this regulatory region, suggesting that multiple consensus sites are required for regulation of the targets of BPC1 (Kooiker et al. 2005). *STK* has been shown to be upregulated about 3 times respect to wt *STK*-expression in the *bpc1* mutant, however without changing the specific expression. Neither are any other obvious phenotypes present in *bpc1* mutant plants (Kooiker et al. 2005). This could be due to the high sequence similarity between *BPC1* and *BPC2/3*, which could have a redundant function with *BPC1*.

Here we describe *bpc1/2/3* single, double and triple mutant characterization, which show a range of phenotypes. Furthermore we show the overexpression of the individual *BPC*'s under the constitutive 35S-promoter. Both in the mutant plants and in plants ectopically expressing *BPC1/2/3* the level of putative target gene expression was assessed using quantitative RT PCR's. Our results suggest that *STK* and *AG* are regulated by *BPC1/2/3*.

## Results

### *bpc1, bpc2* and *bpc3* single mutants

To determine the function of type I BPC proteins, we identified mutants for the *BPC* genes in the SALK-collection. The *bpc1* single mutant did not cause any obvious phenotype except for the upregulation of the expression of genes like *STK*, *INO* and *BPC2* (Kooiker et al. 2005);data not shown). The *bpc2* mutant we analysed (SALK_090810) was identified by PCR and Southern blot (see materials and methods. Similar to the *bpc1* mutant we analysed, this *bpc2* mutant shows an increase in the expression level of *STK* and *INO*, suggesting that *BPC1* and *BPC2* have similar functions.

The *bpc3* mutant that we obtained from the SALK collection (SALK_061981) did not result in a complete knock-out of *BPC3.* In fact the expression of *BPC3* in this mutant was reduced to a level of about 40% of the wild-type expression.
Therefore we used the TILLING line CS89719 which introduces a stop codon at amino-acid 242 leaving a truncated protein where 41 amino-acids are lacking. These 40 amino acids are important for the DNA binding as was shown in yeast 1-hybrid experiments (Meister et al. 2004; Kooiker et al. 2005) and therefore it is unlikely that the formed protein is functional. Nevertheless this mutant does not show any obvious phenotype, like the *bpc1* and *bpc2* mutants.

**bpc1 bpc2 bpc3 triple mutants**

Double mutants did not show a phenotype so we analysed the triple mutant. In the *bpc1 bpc2 bpc3* triple mutants developmental defects have been observed respect to the wild-type, and many of the phenotypes were enhanced when the plants were grown at 28°C. In the triple mutants there is an increase in floral organ number and flowers containing 5 sepals, 5 petals and 7 stamens are frequently seen in the first produced flowers. Later in development the floral organ number is similar to wild-type.
The number of seeds per silique is reduced with respect to wild-type. Analysis of the morphology of the siliques revealed that in some cases the silique consists of 3 instead of 3 fused carpels (Figure 1). The septum in these siliques is not fused properly, probably resulting in a defect in development of the transmitting tract (Figure 1A). Some of the carpels produced as little as 6 seeds due to fertility problems. To investigate if this problem was caused by the female or male gametophyte reciprocal crosses were performed.

When we pollinated wild-type carpels with mutant pollen, it was difficult to obtain 100% fertilized ovules, though repeated pollination resulted in nearly full siliques. On the other hand, when taking wild-type pollen to fertilize mutant ovules, the upper half of the siliques contained 100% fertilized ovules, whereas the lower part of the silique contained a high percent of aborted seeds, probably due to defects in the septum, as described above.
To analyse pollen development, pollen was germinated in vitro and the pollen was divided in 4 different classes, according Lago et al. (2005). Class I corresponds to pollen that germinated and formed a pollen tube more than 3 times the diameter of the grain. Pollen that formed a pollen tube between 1 and 3 times the diameter of the pollen grain, were assigned to class II, class III pollen are

pollen that germinated and formed a short pollen tube of maximal the length of a pollen grain. The class IV pollen did not germinate. The results of these experiments is shown in Figure 1B and 1C. After 24 hours of germination, wild-type pollen contains about 62% of class I pollen tubes, whereas 13% of the pollen are class II pollen. 7% of the pollen grains were classified as class III and the remaining 18% of the pollen did not germinate (class IV). In the *bpc1 bpc2 bpc3* triple mutant only about 12% of the pollen tubes reached stage I, about 10% reach stage II, 15% of the pollen tubes were classified as class III. The remaining 63% of the pollen did not germinate.

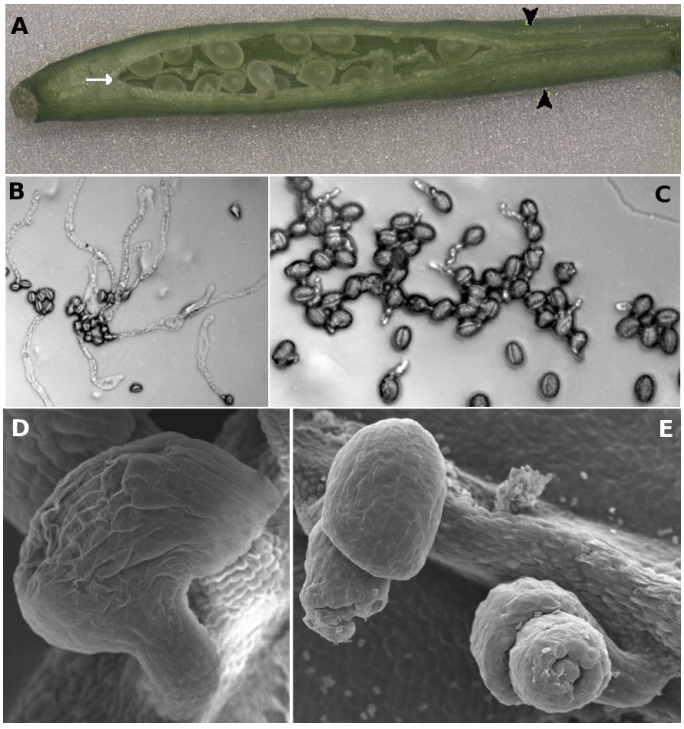


**Figure 1**: Phenotypes of the *bpc1 bpc2 bpc3* triple mutants

**A**: Silique from a triple mutant plant with 3 fused carpels and septum that is not fused properly. The upper valve has been removed and the other two valves are indicated with arrowheads. The white arrow indicates the non-fused septum.

**B:** Pollen tube growth of wild-type pollen after 24h growth on germination medium.

**C:** pollen tube growth of triple mutants after 24h growth on germination medium.

**D:** SEM of a wild-type ovule showing integuments that are grown around the embryo sac.

**E:** SEM of triple mutant ovules showing a block in the growth of the outer integument

Furthermore, 15% of the ovules in the bpc1 bpc2 bpc3 triple mutant show a defect in the integument growth. In wild-type ovules the integuments grow around the nucellus in an asymmetric way leaving an opening at the micropilar end where the pollen tube enters into the embryo sac to fertilize the egg cell and the central cell.

In the triple mutants 15% of the ovules showed integuments that fail to grow in this asymmetric way around the nucellus. In the first place the outer integument stops growing at an early stage, whereas the inner integument and the nucellus continue to develop. The phenotypes are strongly enhanced when the plants are grown at 30°C. The septum is not formed at all and no seeds are formed under these conditions in the triple mutants. In the *bpc1 bpc2 bpc3* triple mutant the first whorl sepals are converted to carpel-like structures bearing ovule like structures. The ovules of these plants have a severe defect in the outer and inner integument growth, ranging from the phenotype observed at normal growth condition (22°C) until the growth of the outer integument into the opposite direction and the presence of stoma on the outer integuments.
We have performed in-situ hybridizations to study the expression of *STK* expression in the *bpc1 bpc2 bpc3* triple mutants. The expression of *STK* in wild-type plants starts at the ovule primordia stage of ovule development where *STK* is expressed in the septum and the ovule primordia (figure 2A). Later in development, *STK* expression is restricted to the ovule, with higher expression in funiculus. In the *bpc1 bpc2 bpc3* triple mutants, the expression of *STK* at the tetrad stage is nearly absent in the septum, as can be seen in figure 2B.

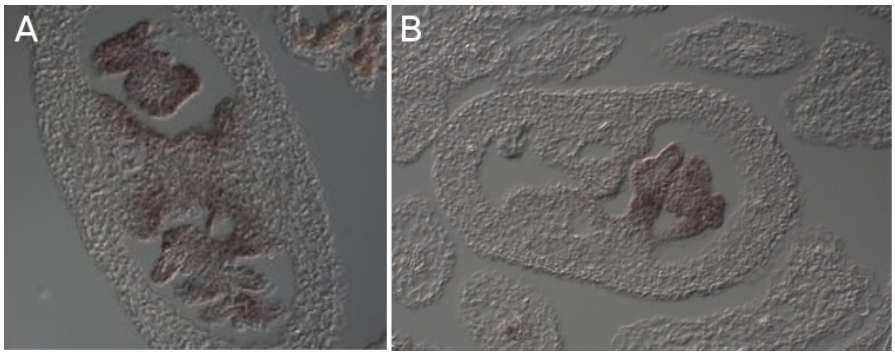


**Figure 2:** in situ hybridization using antisense STK as a probe
**A:** expression of STK in wild-type Arabidopsis ovules and septum.
**B:** expression of STK in ovules of triple mutants *bpc1 bpc2 bpc3*. signal in the septum is nearly absent.

### Overexpression of *BPC1/2/3* genes

In order to obtain some more information about the possible roles of the BPC proteins, we expressed the coding sequence of these genes under the control of the 35S constitutive promoter (Benfey et al. 1990b) and transformed Arabidopsis plants with these constructs. Nineteen independent transgenic lines were selected and *BPC* expression levels were analysed by northern

blot analysis, using RNA extracted from leaves. All plants had high *BPC1* expression levels, and 7 out of 19 plants showed altered floral phenotypes, mainly towards the end of the life cycle of the plant. These defective flowers often do not have sepals and petals, and often only a few stamens are formed. Usually there are more than two carpels, which fail to fuse properly. The ovules within these carpels seem to have a long funiculus and carpelloid structures.


**Figure 3:** late arising flowers of plants expressing the cds of BPC1 under the 35S promoter.
A: sepals are converted into carpel-like organs, whereas second whorl petals are missing. Only one stamen grew and the carpels are not fused.
B: sepals are converted into carpel-like organs bearing ovules, whereas second whorl petals are completely missing. In the third whorl only one stamen grew.

This phenotype resembles the *ap2* mutant. In *ap2* the sepals are converted into carpel-like structures and absence of petals and major part of the stamens do not develop. In *ap2* the repression of *AG* by *AP2* in whorl 1 and whorl 2 is lacking, resulting in the ectopic expression of *AG* in the first two whorls of the Arabidopsis flower (Drews et al. 1991). Ectopic expression of *AG* results in the formation of carpelloid sepals, similar to those found in *ap2* mutants (Mizukami and Ma 1992). These phenotypes are highly similar to the phenotype found in plants overexpressing *BPC1*, *BPC2 or BPC3*, and therefore we performed real-time RT-PCR experiments to assess the relative expression of these genes in our transgenic plants. In order to do this, we amplified the cDNA obtained from leaf and flower RNA, by real-time PCR. As show in Figure 4, the expression of *BPC1* and *AG* seems to be increased 30 and 65 fold respectively in leaves. Though *AG* was published to be flower specific (Bowman et al. 1991b), we observed amplification of *AG* in wt leaves though at a very low level. *AP2* instead showed a decrease in expression levels until a relative expression of 0,4. *STK* expression however was absent in wild-type leaves as well as in our

transgenic lines. However, in the flowers the *STK* expression level was decreased until 0.6, whereas *AP2* expression was decreased until 0,7. To understand if the ectopic carpel was caused by ectopic *AG* expression, or by downregulation of *AP2*, we crossed plants overexpressing *BPC1* with ag3/+ plants, in order to obtain *ag3* mutants that overexpress *BPC1*.

*Ag3* mutants that are overexpressing *BPC1* showed the phenotypes shown in Figure 4. The most striking observation is the elongation of the internode between two flowers as can be seen in Figure 4B and C, a phenotype similar to the one described for *sterile apetala/ag-1* (*sap/ag-1*) double mutants (Byzova et al. 1999). In these mutants new flowers arise in the axis of the second whorl and third whorl sepals, a phenotype we also observed in *ag-3* mutants overexpressing *BPC1*, as can be seen in Figure 4B and D (arrow). Furthermore, in 4 plants out of 8 plants analysed with the light microscope and Scanning Electron Microscope, we observed stigmatic tissue on top of the sepals, as can be seen in Figure 4D, showing that *AG* is not required for the formation of stigmatic tissue found when overexpressing *BPC1*.

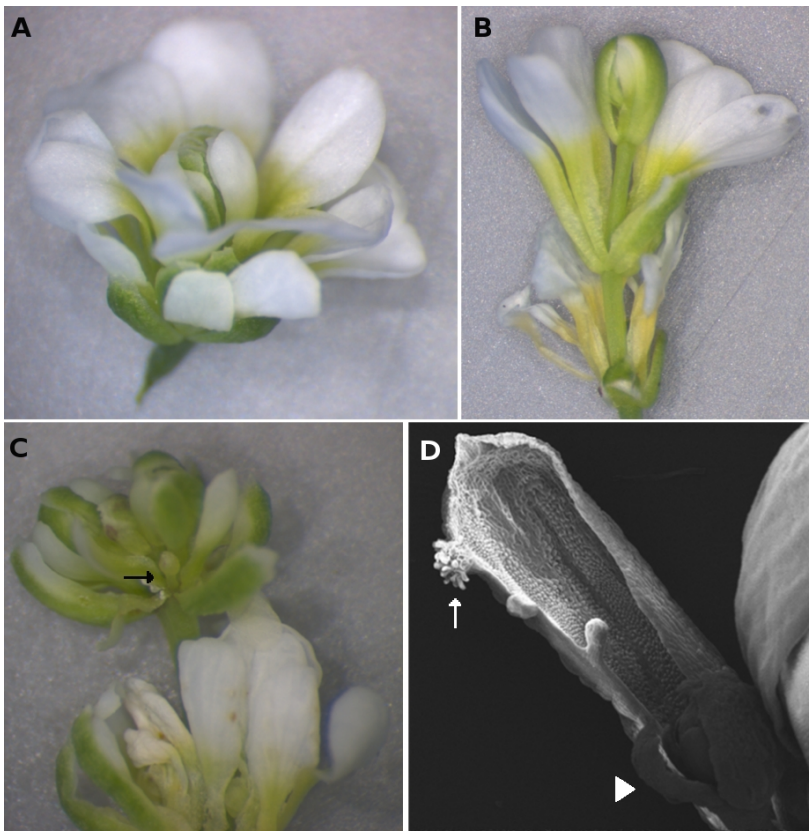


**Figure 4:** Plants ectopically expressing *BPC1* in a *ag3* mutant background.
**A:** *ag3* mutant flower.

**B:***ag3* mutant flower ectopically expressing *BPC1*. Flowers have an elongated pedicel.

**Figure 4** (continued) **C:** Floral buds coming out of the axis of the first whorl sepals (indicated by black arrow).
**D:** SEM from a sepal that shows stigmatic tissue on a first whorl sepal (white arrow). The arrowhead shows a floral bud growing from the axis of a first whorl sepal.

## Conclusion/Discussion

### *bpc1, bpc2, bpc3* single mutants

As was shown by Kooiker et al. (2005), *bpc1* mutants do not show any obvious phenotypes, probably due to redundancy with *BPC2* and *BPC3*. Therefore we analyzed *bpc2* and *bpc3* mutants, found in the SALK collection and TILLING collection respectively. The *bpc2* mutant is a complete knock-out, but does not reveal any obvious phenotypes. This is not a surprising result, seen the homology with *BPC1* and *BPC3*. The *bpc3* mutant is a TILLING mutant that introduces a stop codon at amino-acid 242, truncating the BPC3 protein by 40 amino-acids.
Though this truncated protein could result in a functional protein, this is not likely, since the 40 amino-acids that are missing are highly conserved in BPC proteins throughout different plant species like rice, soy bean and Arabidopsis. In addition, it is reported that this domain is important for DNA-binding, which is an important feature of transcription factors.

### *bpc1 bpc2 bpc3* triple mutants

The *bpc1 bpc2 bpc3* triple mutant shows several phenotypes, of which most can be observed in the flower. Because the consensus BPC binding sequence can be found throughout the entire genome of Arabidopsis it is not surprising to find complex phenotypes, since the expression of many genes might be affected by the lack of the BPC1/2/3 proteins.
Interestingly the observed phenotype is observed mainly in flowers. Flowers that are formed early in development show an increased number of organs, whereas later in development the number of organs that are formed is comparable to wild-type flowers. A difference in defects in early and late arising organs has been reported before in other mutants like *STERILE APETALA (SAP)*, which shows more severe floral phenotypes in later arising flowers (Byzova et al. 1999).

Also pollen is affected in the *bpc1 bpc2 bpc3* triple mutant, resulting in partial sterility at normal growth conditions to complete sterility at 28 degrees. The pollen grains of the triple mutants have a drastic decreased in germination rate, resulting in proper germinating and growing pollen tubes of about 15%, whereas in our experiments this rate in wild-type plants was about 60%.

About 15% of the ovules in triple mutants do not develop as in wild-type and these defects mainly involve the growth of the outer integument, which does not grow asymmetrically as in wild-type ovules, and as a result do not cover the inner integument and embryo sac.
This phenotype is drastically increased when the plants are grown at higher temperatures, where some ovules can be found that have stomata. Also other ovule defects have been observed, like ovules that grow leaf-like structures instead of outer integuments and ovules in which the outer integument grows to cover the funiculus instead of the nucellus.
Similar defects have been reported by (Brambilla et al. 2007), in *stk shp1 shp2 ag/+* mutant plants, where the outer integuments stop growing prematurely, a defect that was also enhanced at higher temperatures, resulting in the absence of the outer integuments and the conversion of the inner integument into carpelloid structures (after stage 12). The outer integument defects could be explained by the lack of *INO* expression. In fact, our triple mutants grown at 30°C highly resemble *ino-4* mutants. *Ino-4* is a weak *ino* mutant, in which the outer integument prematurely stops growing, resulting in ovules that are not completely covered by the outer integument. Confirmation of this hypothesis must be revealed by the analysis of *INO* expression in these mutants (in progress).

**Overexpression of *BPC1/2/3***

The overexpression of the *BPC1*, *BPC2* and *BPC3* genes resulted in very similar phenotypes, which is not surprising, since they show a high degree of similarity. The ectopic carpel-like structures formed in late arising flowers of plants overexpressing *BPC1*, *BPC2* and *BPC3* can be explained by the deregulation of *AP2*. Though the expression of *AP2* is only slightly deregulated in flowers, this deregulation might be more severe in late arising flowers. The expression of *AG* however is not altered in these transgenic plants, but to exclude that *AG* is not required for the phenotype, we crossed the 35S:*BPC1* plants with *ag3/+* plants. Indeed plants homozygous for ag3 that have the 35S:*BPC1* construct are still able to form ectopic stigmatic tissue on the sepals.
The elongation of the internode and the formation of secondary flowers in the axis of sepals and petals is similar to *ag-1 sap* double mutant, however, it is improbable that *SAP* is regulated by the *BPC* proteins, since there are only a few *BPC*-binding sites present in and around the *SAP* gene.
It is interesting to see a similar phenotype both in plants ectopically expressing *BPC* and in *bpc1 bpc2 bpc3* triple mutant plants. A possible explanation could be that the BPC proteins form a multimeric complex, which becomes instable, both when deleting at least three members of the

*BPC* gene family and also when disturbing the balance between protein levels of the individual BPC proteins, by overexpressing one of them. This theory is consistent with the observation of an enhancement of the phenotype of the triple mutants when grown at 28 degrees. The high temperature could destabilize the remaining complex even further.

## Methods

### Plant material

Plants were grown for two weeks in Short-day conditions (8 hours day and 16 hours night) for two weeks and subsequently moved into long-day conditions (16 hours day and 8 hours night) at 22 ˚C or 28˚C.

Mutant lines for BPC2 and BPC3 were obtained from the European Arabidopsis Stock Centre (NASC)(Scholl et al. 2000).

For bpc1 we used the SALK_072966.43.30.x, and genotyping was performed according (Kooiker et al. 2005). We found the bpc2 mutant in the SALK-collection (SALK_090810), and these plants were genotyped by PCR, using the primers AtP437 (5' -AGCCCGGGCATGGATGACGATGGGTTTCG - 3' ) and AtP438 (5' -ATGTCGACTCATCTGATTGTGACGAACTTG- 3') to amplify the wild-type allele and AtP437 and AtP58 (5' - TGGTTCACGTAGTGGGCCATCG - 3') to amplify the mutant allele.

The bpc3 mutant we used came from the TILLING collection ( CS89719), which introduces a stop codon and an AluI site. To genotype these plants we amplified the genomic DNA, using primers AtP725 (5'-GAGTACAAAGAGAGAGAAGTCC-3') and AtP 649 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTATCTGATGGTGACGAACTTATTGG -3') and subsequently digested the PCR product with AluI, resulting in a band of 365bp of the wild-type allele and 125bp + 240bp bands of the mutant allele.

### Microscopy

Clearing of ovules of transgenic plants was performed as described by (Brambilla et al. 2007) and ovules were subsequently observed using a Zeiss Axiophot D1 microscope equipped with DIC optics. Images were captured on an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

Preparation and observation of plant material for scanning electron microscopy was performed as described by Brambilla et al. (2007).

**Expression analysis**

For the in situ hybridization, Arabidopsis flowers were fixed and embedded in paraffin as described previously (Lopez-Dee et al. 1999). Digoxigenin-labeled gene-specific antisense RNA probes were generated by in vitro transcription following the instructions of the in vitro transcription kit (Roche). Hybridization and immunological detection were performed as described previously (Lopez-Dee et al. 1999).

Real-time experiments were performed like previously described by Kooiker et al. (2005).

**Pollen Germination**

Pollen germination experiments were performed as described by Lago et al. (2005).

# Chapter 6

# Concluding remarks and future prospectives

One of the best characterized gene families in Arabidopsis is the MADS-box transcription factor family. This gene family consists of 107 members (Parenicová et al. 2003), which have all been cloned, but only a relatively small part of these gene have been characterized and especially the type I MADS-box genes remain largely uncharacterised. The large scale analysis of expression and two-hybrid analysis described in **chapter 2** gives a good indication of networks within the MADS-box protein family (de Folter et al. 2005). An unknown protein that interacts with a characterized protein, might indicate that these two proteins have a function in the same pathway. Furthermore, the interactions can give clues about the interaction between regulatory networks and diversification of function. An example of the functional diversification are the MADS-box genes that cluster together with the homeotic gene *AGAMOUS*, namely *STK*, *SHP1* and *SHP2*. The proteins encoded by these genes are highly similar and probably are the result of relatively recent duplications. Nevertheless these genes acquired different functions, like control of fruit dehiscence for *SHP1/2* (Liljegren et al. 2000) and control of the development of the funiculus and integuments by *STK* (Pinyopich et al. 2003). But these genes also maintained an important role in the determination of the ovule identity, a function shared with their common interaction partners, namely the *SEP* genes, as described in **chapter 3** (Favaro et al. 2003). The determination of ovule identity by C, D and E type genes seems very well conserved in different plant species, as *fbp7 and fbp11* (D-class genes from Putunia) cosuppression mutants and *fbp2 fbp5* (E-class genes from Petunia) show conversion of ovules into carpel-like structures (Angenent et al. 1995; Vandenbussche et al. 2003), just like their counterparts in Arabidopsis. Also in Rice the D-class function seems to be conserved, since the *osmads13* mutant shows a disruption of ovule development, converting these organs into carpel-like structures (Dreni et al. 2007). It would be interesting to see if the organ identity complexes formed in Arabidopsis and Petunia are formed also in monocot species as rice. Putative orthologs of the SEP genes in rice are OsMADS1, OsMADS5, OsMADS24, OsMADS34 and OsMADS45, however up till now only OsMADS1 and OsMADS5 have been characterized, and osmads1 mutants show a phenotype similar to sep1 sep2 sep3 triple mutants in Arabidopsis (Agrawal et al. 2005). However expression of the B, C and E class genes do not seem to overlap and moreover these proteins have never shown to interact (for a review see Kater et al. (2006), characteristics that are essential in the Arabiopsis model. Therefore it might be that the true orthologs of the SEP genes are OsMADS24 and OsMADS45, based on phylogenetic data, interaction partners and expression data, as suggested by Kater et al. (2006).

It remains to be proven if these proteins can form ternary complexes in yeast, like the Arabidopsis complexes that determine diverse floral organ identities.
Furthermore it remains to be seen if these ternary complexes are really formed in planta, since these complexes have never been identified in vivo. However besides the formation of multimeric complexes in yeast (as firstly described by Egea-Cortines et al. (1999)), also genetic data like the ectopic co-expression of A+B+E class genes, B+C+E class genes and C+D+E class genes suggest that these complexes could be formed in plants (Honma and Goto 2001; Pelaz et al. 2001a; Battaglia et al. 2006). Final proof could be obtained by performing experiments like in planta co-immunoprecipitations, TAP tag technology, or a combination of Fluorescence life-time immaging (FLIM) and the bimolecular fluorescence complementation (Bracha-Drori et al. 2004).
Though much is known about the interaction partners of many MADS-box proteins and the loss of function of several MADS-box genes, little is known about the targets of MADS-box genes. Recently however interesting articles have been published that describe the characterisation of targets of MADS-box genes using Chromatin Immuno-precipitations (ChIP) or Chromatin Affinity purifications (ChAP). In these experiments antibodies directed against AG were used (Gómez-Mena et al. 2005) or antibodies directed against a fluorescence tag was used (de Folter et al. 2007; Gregis et al. 2008). Though this method requires the verification of the maintenance of the biological function of the tagged protein, this is a very powerful tool to identify putative targets of any gene, without producing antibodies against each protein. These tools become even more powerfull if combined with microarrays (chip on ChIP) (Lieb 2003) or high-throughput sequencing methods like the Ilumnia sequencing technology (ChIP-seq, Robertson et al. (2007), or QuEST (Valouev et al. 2008).

It is interesting to see that a MADS-box protein can determine the identity of different organs, simply by interacting with different MADS-box proteins and it evidences that a correct regulation of these genes is very important, since the misexpression of these MADS-box genes may lead to the formation of the incorrect protein complexes, which may lead to the homeotic conversion of one organ into another, as was shown in **chapter 3** for the ectopic expression of SHP1/2, STK and AG resulting in the conversion of sepals into carpelloid structures (Favaro et al. 2003).
Though the importance of this tight regulation is obvious, not much is known about the regulation of MADS-box genes in general. In **chapter 4** the regulatory region of *STK* was used in a one-hybrid screening to identify factors controlling *STK* expression. The most interesting protein that was

found to bind this regulatory region was BASIC PENTACYSTEINE 1, which was able to bind several different regions of the regulatory region of *STK*. Using the Tethered Particle Motion (TPM) technique it was shown that this protein was able to change the secondary structure of the DNA in this in vitro experiments, by binding to multiple binding sites (Kooiker et al. 2005).
Functional analysis of this gene by studying the *bpc1* mutant, confirmed the interaction of this protein to the *STK* regulatory region, since in this mutant the expression of *STK* changed, however other obvious phenotypes were absent, probably due to redundancy with other genes from this small gene family. Subsequent analysis of *bpc1 bpc2 bpc3* triple mutants (**chapter 5**) resulted in more convincing phenotypes, mainly in the disruption of integument growth and lack of pollen tube growth, confirming the redundancy between *BPC1/2/3*. It is interesting to see that this phenotype is enhanced at higher temperatures, and a possible explanation for this could be that the other BPC proteins are still able to form functional complexes, but that these complexes are less stable and are disrupted at higher temperatures. The target genes that are responsible for this phenotype are not clear yet, but obvious candidates are *STK*, *INO* and *AG*, all three of these genes contain several *BPC*-target sequences and are known to play an important role in the development of ovules and integuments (Brambilla et al. 2007). Also overexpression of *BPC1*, *BPC2* and *BPC3* indicate that they regulate *AP2* and *AG*, since these plants show an up regulation of *AG* of 65 times and a down regulation of 2.5 fold of *AP2*.
Experiments are in course to identify these target genes, in-situ hybridisations and crossing the triple mutants with promoter-GUS lines. However it is still probable that even further redundancy exists between *BPC1/2/3* and the other members of the *BPC* gene family. To answer this question, septuple mutants are being made and in the meantime promoter-GUS studies are being performed, where the BPC-binding sites are destroyed by point mutations, resulting in the lack of binding of the BPC proteins.
Some questions that remain to be addressed are the way of functioning of the BPC proteins. How do they regulate the gene expression? Is the loop formation, which we demonstrated in vitro, also taking place in vivo and is this important for the function of BPC's? Do the BPC proteins have more features in common with GAGA-Factor (GAF) from Drosophila besides the binding to GA-rich sequences? Do they play a role in chromatin remodelling?

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# Summary

During the life cycle of plants, it is important that the right genes are expressed in the right moment and at the right place. If we are talking about responses to stress, responses to change in day-length or the normal housekeeping genes. All genes are regulated by transcription factors, which allow the right expression at the right moment and place. There are many transcription factors present in plants and one of the “master regulators” are the transcription factors of the MADS-box gene family. These MADS-box genes are involved in many different processes during the life cycle of plants, varying from determination of flowering time to determination of organ identity.
The sequencing of the entire genome revealed the presence of 107 MADS-box genes in Arabidopsis, and though many mutants revealed the function of many of these mutants, a large number of these MADS-box genes still have an unknown function.
One important step in the development of Arabidopsis is ovule formation, which in Petunia is controlled by the MADS-box genes *FBP7* and *FBP11*. During this thesis the aim was to study the Arabidopsis homogue of *FBP7* and *FBP11*, namely *SEEDSTICK* (*STK*). Looking at it's interaction partners and regulators of the gene.
**Chapter 1** describes the different stages in ovule development and the link with the ovule identity and MADS-box genes. The determination of organ identity, which is lost in the *stk shp1 shp2* triple mutant as well as *sep1*/+ *sep2 sep3* mutants, is controlled by multimeric MADS-box complexes. Other groups showed the formation of multimeric complexes between for example the MADS-box proteins SEPALLATA3, AGAMOUS, PISTILLATA and APETALA3.
In **chapter 2** a matrix based two-hybrid interaction study is are described between all 107 MADS-box genes. Using the yeast two-hybrid technique, putatively all interactions between MADS-box proteins were revealed, resulting in a comprehensive interaction map of MADS-box proteins. These interactions might give a clue on the function of these proteins in a specific developmental program of unknown proteins. Proteins that seem to participate can function as a sort of hub between different developmental processes and link these processes. Several of these MADS-box proteins interact with many other MADS-box proteins. These proteins (for example SEP3) are important for the development of many different organs, and it is the partners that specify the function in different situations.
Since it has been shown that MADS-box proteins form higher order complexes, in **chapter 3** the ovule identity proteins STK, SHP1 and SHP2 were tested in three hybrid experiments which

showed the formation of higher order complexes between these proteins in the presence of SEP3. The existence of these higher order complexes was supported by genetic experiments, where the *shp1 shp2 stk* triple mutants lead to the conversion of ovules into carpeloid structures. Ovule development was also completely disrupted in plants that were heterozygous for *sep1* and homozygous for *sep2* and *sep3*, showing the lack of formation of an efficient ovule identity complex. Ectopic expression of the ovule identity genes resulted in the formation of ectopic ovules on sepals, always in combination with ectopic carpelloid structures.

This experiment shows the importance of a tight regulation of the ovule identity genes and in **chapter 4** the identification of regulators of the "master regulators" was described. Using a yeast one hybrid screening BASIC PENTACYSTEINE 1 was identified as a protein that interacts with several regions in the *STK* regulatory region. Using electro mobility shift assays (EMSA) the interaction between the *STK* regulatory region and BPC1 was confirmed and a consensus binding sequence for BPC1 was determined. Using the tethered particle motion (TPM) technique we reveiled the capacity of BPC1 to change the conformation of the DNA upon binding. *bpc1* mutants resulted in the upregulation of the *STK* transcript, showing that BPC1 acts as a repressor of the *STK* gene.

However since the single mutant did not show any obvious phenotypes, triple mutants were obtained, and described in chapter 5, crossing *bpc1* mutants with *bpc2* and *bpc3* mutants. The triple mutants show several phenotypes. For example the pollen grains have a very low germination rate and the integuments fail to grow around the nucellus in many cases. Also ectopic expression of *BPC1*, *BPC2* and *BPC3* is described in this chapter, resulting in the formation of ectopic carpelloid structures on the sepals. In the leaves of these plants the expression of AG is upregulated about 60 times, and *AP2* is downregulated about 2,5 times, suggesting a regulation of these genes by the BPC proteins.

# Samenvatting

Gedurende de levenscyclus van planten, is het belangrijk dat de juiste genen op het juiste moment en op de juiste plaats tot expressie komen. Dit geldt met name voor genen die de ontwikkeling van een plant reguleren. Genen worden gereguleerd door transcriptie factoren, die verantwoordelijk zijn voor expressie van de juiste genen op de juiste plaats en tijd. Met name planten bezitten een grote hoeveelheid genen die voor transcriptie factoren coderen. Een groep transcriptie factoren die met name bloei inductie en bloemontwikkeling reguleren behoren tot de MADS-box familie.
Door het bepalen van de basenpaar volgorde van het hele genoom van de model plant Arabidopsis thaliana konden er via de analyse van deze genoom sequentie 107 MADS-box genen geïdentificeerd worden. Tot heden zijn de meeste van deze MADS-box genen nog niet functioneel gekarakteriseerd. Het onderzoek dat staat beschreven in dit proefschrift, is met name gericht op het Arabidopsis gen *SEEDSTICK* (*STK*) dat de zaadknop vorming reguleert. **Hoofdstuk 1** beschrijft de verschillende stadia van de zaadknop ontwikkeling en hoe de bloemknop identiteit wordt bepaald door MADS-box genen. Uit deze studie wordt duidelijk door een combinatie van genetische experimenten en eiwit interactie bepalingen, dat de MADS-box eiwitten die zaadknop identiteit bepalen (STK, SHP1 en SHP2) complexen vormen met de SEPALLATA eiwitten SEP1, SEP2 en SEP3. In **hoofdstuk 2** is een matrix gebaseerde “two-hybrid” interactie studie beschreven tussen alle 107 MADS-box eiwitten. Gebruik makend van de gist “two-hybrid” techniek, zijn praktisch alle interacties tussen 107 MADS-box eiwitten getest, hetgeen een alomvattende interactie kaart van MADS-box eiwitten opgeleverd heeft. Deze interacties kunnen een indicatie geven voor de functie die deze eiwitten in specifieke ontwikkelingsprogramma's kunnen spelen. Sommige eiwitten kunnen verschillende ontwikkelingsprocessen aan elkaar verbinden door te interacteren met verschillende eiwitten uit verschillende processen. Een aantal MADS-box eiwitten interacteren met veel andere MADS-box eiwitten. Deze eiwitten (zoals bijvoorbeeld SEP3) zijn belangrijk voor de ontwikkeling van meer organen en het zijn waarschijnlijk hun partners die ervoor zorgen dat deze eiwitten hun specifieke functie uitvoeren in verschillende situaties.
Omdat MADS-box eiwitten complexen vormen, zijn in **hoofdstuk 3** de three-hybrid experimenten beschreven die aantonen dat ook de eiwitten die de zaadknop de juiste identiteit geven hogere order complexen vormen, met behulp van SEP3.
Het bestaan van deze hogere order complexen is bevestigd met behulp van genetische experimenten, waar de *shp1 shp2 stk* mutant leidt tot de conversie van zaadknoppen tot stamper-

gelijkend weefsel. De zaadknop ontwikkeling was ook compleet verstoord in planten die heterozygoot zijn voor de *sep1* mutant en homozygoot voor *sep2* en *sep3*, hetgeen het ontbreken van een effectief complex van eiwitten die de identiteit van de zaadknop bepaalt aantoont. Overexpressie van de zaadknop identiteits-genen resulteerde in de formatie van ectopische zaadknoppen op kelkbladen, hetgeen altijd gepaard gaat met ectopisch stamper-gelijkend weefsel. Deze experimenten tonen aan dat een strikte regulatie van zaadknop identiteitsgenen heel belangrijk is, en in **hoofdstuk 4** is de regulatie van deze "master regulators" beschreven. Gebruik makend van de gist one-hybrid techniek, is BASIC PENTACYSTEINE 1 (BPC1) geïdentificeerd als een eiwit dat in staat is om op verschillende plaatsen interactie aan te gaan met de promotor van *STK*. Door gebruik te maken van "electro mobility shift assays" (EMSA) is de interactie tussen de promotor van *STK* en BPC1 bevestigd en is een consensus sequentie voor de binding van BPC1 aan het DNA bepaald. Tethered particle motion (TPM) techniek is vervolgens gebruikt om aan te tonen dat BPC1 in staat is om de structuur van het DNA te veranderen door zich aan dit DNA te binden. De *bpc1* mutant resulteerde in een hogere expressie van het *STK* transcript, hetgeen aantoont dat BPC1 een repressor is van het gen *STK*. Echter, gezien het feit dat er geen voor de hand liggend fenotype te zien was, zijn er drie-dubbel mutanten gemaakt, door *bpc1*, *bpc2* en *bpc3* mutanten te kruisen, zoals beschreven in **hoofdstuk 5**. Deze drie-dubbel mutanten tonen verschillende fenotypes. Bijvoorbeeld het stuifmeel heeft een zeer lage kiemkracht en de integumenten van de zaadknop is vaak niet in staat om over de nucellus heen te groeien. In hoofdstuk 5 is ook de overexpressie van *BPC1*, *BPC2* en *BPC3* beschreven, hetgeen leidde tot de formatie van ectopische stamper-gelijkend weefsel op kelkbladen. In de bladen van deze planten is de expressie van *AG* ongeveer 60 keer hoger dan de expressie in normale planten, en de expressie van *AP2* is ongeveer 2,5 keer lager dan de expressie in normale planten.

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# Nawoord

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